THE REQUIREMENT OF THE TURKEY POULT FOR VITAMIN B_c[1]

LUTHER R. RICHARDSON, ALBERT G. HOGAN AND HARRY L. KEMPSTER
Departments of Agricultural Chemistry and Poultry Husbandry, University of Missouri, Columbia

ONE FIGURE

(Received for publication June 7, 1945)

Several feedstuffs, such as dried skim milk or alfalfa leaf meal, are regarded as important or even critical items in the ration of newly hatched turkey poults, but in recent years the supply has not been equal to the demand. An attempt was made in this laboratory to formulate satisfactory rations that contained none of these constituents, and although some degree of success was attained the poults made only moderate gains in weight. It was suggested that the slow rate of growth was due to an amino acid deficiency, and, to test this hypothesis, the vegetable protein concentrate in one of the practical rations was replaced by casein and gelatin. When this change was made the ration was a complete failure and the poults assumed a typical, and abnormal, posture. Such results led us to a more extended study of the problem.

EXPERIMENTAL

Day-old Bronze turkey poults were transferred to small brooders provided with automatic electric heating units. The temperature varied from 90° to 95°F. At the age of 2 weeks they were transferred to another room, maintained at a temperature of 80° to 85°F. The experimental rations, and water, were constantly available. It may be of some interest to mention here that practical turkey growers often state that poults must be taught to eat, but in our experience this is unnecessary. If the environment and ration are suitable, and if the feeders are properly constructed, normal poults begin to eat promptly. The poults were weighed once a week, and they were examined frequently for abnormalities.

[1] Contribution from the Department of Agricultural Chemistry, Missouri Agricultural Experiment Station, Journal Series no. 980. Aided by a grant from Parke, Davis and Company.

Copyright, 1945
The Wistar Institute of Anatomy and Biology.
All rights reserved.

The ration in use when this type of paralysis first appeared was modified slightly in later trials, but most of the modifications had no significance and for the present only Ration A will be described. While these studies were under way we were also investigating the response of turkey poults to synthetic diets. Poults grow remarkably well on these diets when a liver extract is included, and therefore various liver fractions were added to the synthetic diets in an attempt to attain

TABLE 1

Rations deficient in vitamin B_c.

CONSTITUENTS	RATION A	RATION B
	gm.	*gm.*
Yellow corn	49.50	....
Wheat bran	5.00	....
Wheat middlings	15.00	....
Steamed bone meal	2.00	....
$CaCO_3$	1.00	....
NaCl	0.46	....
$MnSO_4.4H_2O$	0.04	....
Casein	20.00	35.00
Gelatin	5.00	10.00
Cerelose	....	32.00
Cellulose	....	3.00
Lard [1]	2.00	10.00
Mineral salts	....	5.00
Liver extract fraction B [2]	....	5.00
Additional vitamins	see footnote [3]	see footnotes [3, 4]

[1] Carrier for fat-soluble vitamins.

[2] This fraction contains only a trace of vitamin B_c.

[3] Vitamin A	2000 I.U.	Alpha-Tocopherol	2.5 mg.
Vitamin D	283 A.O.A.C Units	2-methyl-1, 4-napthoquinone	2.5 mg.
Thiamine	1 mg.	Ca-Pantothenate	2.0 mg.
Riboflavin	1 mg.	Nicotinic acid	5.0 mg.
Pyridoxine	1 mg.	Choline	200.0 mg.

[4] Inositol 100 mg., p-aminobenzoic acid 100 mg., biotin 10 μg.

All vitamins except A and D were supplied by Merck and Co., Rahway, N. J.

greater concentration of the active agent. One [2] of the extracts was described to us as the filtrate, or by-product, from the first stage in the isolation of vitamin B_c (Pfiffner et al., '43). This fraction contains only a trace of vitamin B_c as indicated by microbiological assay, and when it was substituted for the original liver extract, as in Ration B, the poults developed cervical paralysis even more quickly than they did on Ration A. The composition of the two rations is shown in table 1 and

[2] Provided by Dr. J. J. Pfiffner, of Parke, Davis and Company.

the observations on the poults are summarized in table 2. Several abnormalities developed, but the most characteristic symptom was a spastic cervical paralysis. At the onset the neck is extended and rigid, as in a turkey that has been alarmed and is searching for the source of danger. The attacks are at first intermittent, but later they become continuous. The wings are slightly drooped and quiver, the poult chirps continuously as if in pain, and there may be some diarrhea with the excreta thin and white. The condition terminates in death within 24 to 36 hours unless vitamin B_c is provided during the early stages.

There were ten poults in the first trial with Ration A, and it will be noted that five of them developed cervical paralysis. None of these recovered and all of them died in a day or two. Four more died with no

TABLE 2

Vitamin B_c deficiency in turkey poults.

RATION	POULTS PARALYZED			POULTS NOT PARALYZED					
				Died			Survived		
	No.	Age	Wt.	No.	Age	Wt.	No.	Age	Wt.
		weeks	*gm.*		*weeks*	*gm.*		*weeks*	*gm.*
A 1	5	7	765	4	4	81	1	14	1880
2	15	3.5	240	12	2.5	153	4	6	552
B	9	2.5	143	2	2	102	0		
B plus B_c conc.	0	..	...	0	..	..	2	4	498

[1] The mortality is 100% unless vitamin B_c is provided.

characteristic symptoms, and one survived for 14 weeks with no symptoms.

In subsequent trials there were thirty-one poults, and fifteen of them developed the characteristic symptoms. None of these survived except a few which were treated in the early stages with some source of vitamin B_c. Twelve died without characteristic symptoms, and four survived for 6 weeks when they were discarded. It will be noted that in trial 1 the poults developed the symptoms at a later age than in trial 2, presumably because the mothers of the poults had consumed different types of rations. The poults in trial 1 were hatched in October, and their mothers had probably been on range of some kind and had consumed a considerable quantity of fresh forage, which we believe is at least a fair source of vitamin B_c. The poults in trial 2 were hatched in the early spring, and one might assume that the hens that laid the eggs from which they were

hatched had not had access to fresh forage for some weeks. If this explanation is correct the poults were partly depleted of vitamin B_c when hatched. The rate of growth on Ration A was lower than that of poults on our better rations but it was not markedly subnormal.

Two poults were given Ration B with a supplement of a highly concentrated preparation of vitamin B_c.[2] They grew at a normal rate but more extensive studies must be carried out before one can be certain

TABLE 3

Curative treatment of cervical paralysis.

RATION	TREATMENT		NUMBER TREATED	NUMBER THAT RECOVERED	NUMBER DYING AFTER RECOVERY
A (and modifications)	Yeast and milk		2	2	2
	Dried yeast	2 gm.	1	0	
	Yeast eluate	1 gm.	3	2	
	B_c concentrate	105 μg.	1	0	
		210 μg.	4	2	
	Crystalline vitamin B_c	100 μg.	4	1	
		300 μg.	1	0	
	Intraperitoneal injection of crystalline vitamin B_c	50 μg.	2	2	
		100 μg.	2	2	
B	B_c concentrate	210 μg.	3	2	1
	Crystalline vitamin B_c	100 μg.	1	1[1]	
	Crystalline vitamin B_c, 50, 100, and 50 μg. on successive days		1	1[2]	
	Total		25	15	3

[1] Recurrence in 6 days.
[2] Recurrence in 7 days.

that their diet was entirely adequate. Our experience with the concentrate gave no reason to infer that it contained any vitamin other than B_c which is required by the poult. This inference is supported by the fact that chicks which received Ration B supplemented with crystalline vitamin B_c[2] grew at a rate which exceeds that of any current growth standard for the breed employed. Eleven poults received Ration B alone, with no supplements, and nine of them developed cervical paralysis, at an average age of 2.5 weeks. The other two died with no specific symptoms. The results of the various curative treatments are described in table 3.

[2] Provided by Dr. J. J. Pfiffner, of Parke, Davis and Company.

Table 3 presents the results of the curative treatments. Two poults were given a suspension of yeast in milk and both recovered, but they died not long afterward. It is assumed that the suspension contained enough vitamin B_c to give temporary relief, but not enough to restore the poults to a normal physiological condition. One poult was given 2 gm. of dried yeast but it did not recover. Three poults received an eluate of a fuller's earth adsorbate of yeast extract, and two recovered. Eight poults received the vitamin B_c concentrate, and four recovered. Eleven poults were treated with the crystalline vitamin and seven recovered. It will be noted that one of these received three doses, on 3 successive days, before it recovered completely. It is possible that some of the other four would have survived if they had been treated with similar repeated doses. Four of the eleven received the vitamin by intraperitoneal injection and all four recovered. Of the two poults which received 100 μg. of vitamin B_c by intraperitoneal injection, one recovered in 4 hours, the other in 6 hours. A third poult received 50 μg. at 10:00 A.M. There was marked improvement at 5:00 P.M. but the symptoms of deficiency had not entirely disappeared. Recovery was complete by the next morning. The fourth poult received the same quantity of the vitamin at 2:45 P.M. There was no definite improvement at 5:00 P.M. but it was normal the next morning. We are of the opinion that the vitamin is more effective when injected than when it is administered orally, and that curative treatment under our experimental conditions is entirely effective if initiated promptly but less effective when delayed. There were no cases of spontaneous remission, and the cures induced by the crystalline preparation leave no doubt that the disease was caused by a deficiency of vitamin B_c.

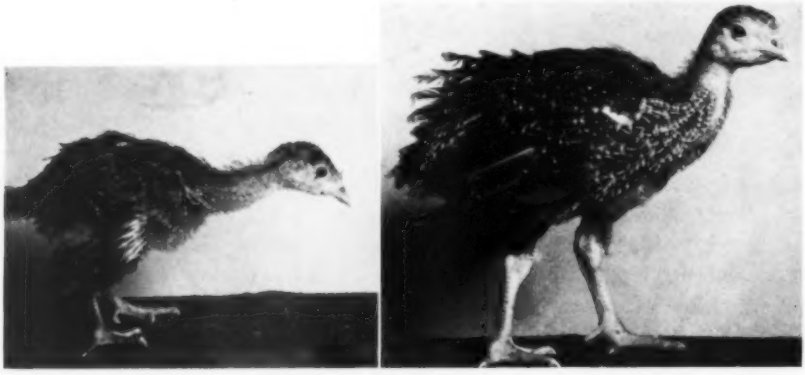
According to Hogan and Parrott ('40) and O'Dell and Hogan ('43) chicks become anemic when they consume diets that are deficient in vitamin B_c; therefore red cell volume was determined in some of the affected poults. The volume of erythrocytes tended to be low, but none of the poults was markedly anemic.

A typical example of a B_c deficiency is illustrated in figure 1. This same type of paralysis has been observed occasionally in chicks which survive for some time after they become anemic.

Distribution of vitamin B_c

Our data on this point are limited, but it is clear that the vitamin is present in some of the feedstuffs in only small quantities. Ration A for example contains 51.5% of yellow corn, 5% of wheat bran, and 15% of

wheat middlings, but some poults, when fed this mixture, gave evidence of a deficiency within 3 weeks. A considerable number of poults received a ration, not previously mentioned, which contained 22% of yellow corn, 3% of wheat bran, 9% of wheat middlings, and 15% of soybean meal, and approximately 8% of these poults developed deficiency symptoms, at ages varying from 15 to 24 days. Soybean meal is not an excellent source of vitamin B_c but it probably contains more than is present in corn or the wheat by-products. It has been observed (unpublished) that anemia in chicks is delayed, but not prevented, by including 2% of high grade alfalfa meal in the ration.



Diet B
Age, 4 weeks Weight, 202 gm.

Adequate diet
Age, 4 weeks Weight, 510 gm.

Fig. 1 The posture of the poult which received Diet B is typical of a deficiency of vitamin B_c.

One poult, not described in this report, developed typical symptoms and died on a simplified ration which contained 5% of dried Strain G yeast. One may reasonably assume that vitamin B_c deserves some consideration in practical turkey production.

SUMMARY

Turkey poults develop a spastic type of cervical paralysis on rations that are severely deficient in vitamin B_c, but which contain reasonable amounts of all other required vitamins.

The poults which were examined had a lower red cell volume than is normal but none were markedly anemic.

Remission of the symptoms was obtained by the administration of crystalline vitamin B_c.

Some of the commonly used feedstuffs are poor sources of vitamin B_c.

LITERATURE CITED

HOGAN, A. G., AND E. M. PARROTT 1940 Anemia in chicks caused by a vitamin deficiency. J. Biol. Chem., vol. 132, p. 507.

O'DELL, B. L., AND A. G. HOGAN 1943 Additional observations on the chick antianemia vitamin. J. Biol. Chem., vol. 149, p. 323.

PFIFFNER, J. J., S. B. BINKLEY, E. S. BLOOM, R. A. BROWN, O. D. BIRD, A. D. EMMETT, A. G. HOGAN AND B. L. O'DELL 1943 Isolation of the antianemia factor (vitamin B_c) in crystalline form from liver. Science, vol. 97, p. 404.

VITAMIN A AND CAROTENE IN THE NUTRITION OF THE GUINEA PIG [1]

LILLIAN S. BENTLEY AND AGNES FAY MORGAN
Laboratory of Home Economics, University of California, Berkeley

ONE FIGURE

(Received for publication April 23, 1945)

The nutritional needs of the guinea pig are still largely unknown in spite of much effort in several laboratories to determine them. The purified diets used for rats and dogs are not satisfactory for guinea pigs, and even the semipurified diets frequently used are only partly successful.[2] To study the guinea pig's response to vitamin A and carotene, the diet must be free of both compounds; thus a suitable semipurified diet had to be devised. In this investigation, therefore, it was necessary first to obtain a diet that was adequate when supplemented with vitamin A, and second to establish a means of recognizing the deficiency in the guinea pig.

Boock and Trevan ('22) investigating the cause of the death of guinea pigs on a diet of bran, oats, mangolds, and water found great improvement in all except one animal when they were given 0.5 ml. of cod liver oil. On a diet of bran and oats, caseinogen, salts, paper and mangolds, two guinea pigs began to lose weight at the end of a month and one developed a cloudiness of the cornea and keratomalacia. Wolbach and Howe ('28) used a diet of casein 15%, starch 74%, butterfat 6%, salts 3%, and yeast 2%; filter paper and distilled water ad libitum, supplemented by orange juice. When lard was substituted for the butter and the amount of orange juice reduced, the only external signs of vitamin A deficiency were cessation of growth and loss of weight. The animals died before typical xerophthalmia was produced.

Though Manzi ('35) attributed loss of hair, eczema of the face, and abortion to vitamin A deficiency, xerophthalmia was observed in only one of his twenty-three guinea pigs. The diet used may have been deficient in other factors, possibly some of the B vitamin complex, and

[1] This study was supported by a grant from Swift and Co., Chicago.

[2] It has been reported that guinea pigs require three unknown dietary essentials (Woolley, '42). No attempt was made in this study to verify these claims or to identify the unknowns.

the eczema and loss of hair could have been the result of such deficiency. Furthermore the animals used had passed their most rapid growth period, and thus would have been less susceptible to a deficiency of vitamin A. Hetler ('34) fed guinea pigs the vitamin-A free diet used in her study of the deficiency in monkeys. Three of the guinea pigs died within a month without developing eye lesions. Three others that had received this diet plus rolled oats ad libitum lived 6 to 9 weeks but, during the last 2 or 3 weeks lost weight rapidly and developed eye lesions. The symptoms observed were dryness and scaliness of the eyelids and cloudiness of the corneas. It was concluded that the guinea pig may react to the deficiency in the manner observed in other species.

Since in all other species studied a deficiency of vitamin A produces xerophthalmia, it seemed unlikely that typical xerophthalmia could not be produced in guinea pigs by means of a vitamin-A-deficient regime. Therefore the attempt was made to verify this supposition.

METHODS

Six semipurified diets and a stock diet were employed in studying vitamin A deficiency, the storage of vitamin A, the utilization of carotene for vitamin A storage, and the adequacy of the preformed vitamin and provitamin during reproduction.

The stock diet

The diet used for the guinea pig colony was a commercial feed [3] composed of ground grains, bran, molasses, soy bean, alfalfa, linseed and bone meals, salt and dried whey. This feed when supplemented with ascorbic acid-fortified orange juice and carotene in oil had been found satisfactory for both growth and reproduction.

The semi-purified diets

The simplified diet, diet C, eventually used, contained, in per cent, casein 25, cornstarch 40, hydrogenated cottonseed oil 3.5, salts [4] 4, K_2CO_3 0.5, wheat germ 10, yeast 10 and bran 7. When supplemented with 6 ml. lemon juice containing 1 mg. ascorbic acid per milliliter three times a week, and with an adequate amount of carotene or vitamin A this diet allowed normal growth of young guinea pigs. Diets I and II were

[3] Globe A — 1 rabbit pellets; Globe Grain and Milling Co., Oakland, California. The composition was as follows: crude protein 15, fat 2.5, fiber 19, ash 10, added salts 1, carbohydrates 52.5%, respectively; thiamine 0.28, riboflavin 0.65 and carotene 0.58 mg. %, respectively.

[4] Hubbell, Mendel, and Wakeman ('37).

tried earlier. These contained larger and smaller proportions of casein (30 and 20% and a lower level of salts (2% with no additional K_2CO_3)). These diets proved inadequate, resulting in poor consumption and failure of growth. Daily addition of 1 ml. cottonseed oil alone or containing 1 mg. mixed tocopherols [5] did not improve the performance on these diets but the increase in salt content from 2 to 4.5% allowed normal growth in the young guinea pigs, 31 gm. gain weekly for 6 weeks (fig. 1).

The modification of diet C, aimed at making it low in vitamin A by use of hot-alcohol extracted casein, was designated diet G. This change in the casein was sufficient to bring about deficiency signs and death when the animals were transferred from C to G. Two other modifications were used, in that carotene-free grass [6] residue or additional cornstarch were substituted for the wheat germ in the diets designated A and B. A concentrated carotene-free water extract of the dried grass was also fed along with these diets at first but these grass preparations were found to have no effect upon growth and were later discarded.
The vitamin A supplement used was a solution of the vitamin [7] in cottonseed oil; the carotene was a carotene-oil concentrate [8] which was assayed for vitamin A value by the U.S.P. biological procedure.

Procedure

The animals were, for the most part, produced in the laboratory colony. They were caged in groups or reared in open runs on shavings. Most of the guinea pigs were placed on the experimental diets when they weighed 200 to 300 gm., at 3 to 5 weeks of age.

The vitamin A content of the livers was determined by the method of Davies ('33) with a few minor changes. The blue color developed with the reagent (Koehn and Sherman, '40) was read in an Evelyn photoelectric colorimeter, and the vitamin values were calculated by comparison with standard curves for crystalline vitamin A.[9] The serum vitamin A was determined by the method of Yudkin ('41). The volume

[5] Concentrate of natural mixed tocopherols, 40%. This was kindly supplied by K. Hickman of Distillation Products, Inc., Rochester, N. Y.

[6] Cerophyl, produced by Cerophyl Laboratories, Inc., Kansas City, Missouri.

[7] This was distilled from fish liver oil and was kindly supplied by K. Hickman of Distillation Products, Inc., Rochester, N. Y. The oil contained 150 mg. of vitamin A per gram as determined chemically.

[8] This concentrate, a product of General Biochemicals, Inc., Chagrin Falls, Ohio, contained 2 mg. carotene per ml.

[9] This was generously supplied as vitamin A alcohol by K. Hickman of Distillation Products, Inc., Rochester, N. Y.

was adjusted for reading with the 6 ml. window of the Evelyn photoelectric colorimeter.

SYMPTOMS OF VITAMIN A DEFICIENCY

The signs of deficiency developed within 2 to 3 weeks. Weight loss and sensitivity of the eyes to light were the first symptoms. Swelling and incrustation of eyelids, keratinization, and corneal destruction by infection were seen in the animals that survived 4 to 6 weeks.

Unless the symptoms of the deficiency were recognized early and therapy instituted at once, the animals could not be saved. Four of five animals died shortly after an attempt was made to cure the deficiency by giving vitamin A or carotene. One of those in the first group (209, fig. 1) responded to the therapy. After the symptoms had been cleared

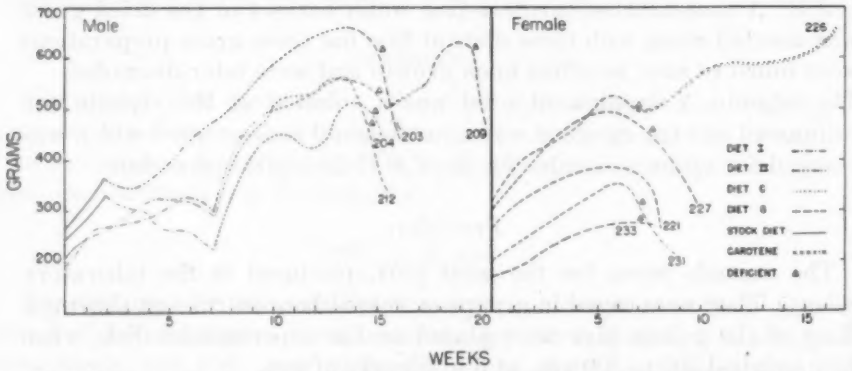


Fig. 1 The growth of male and female guinea pigs on semipurified diets with and without carotene.

up the carotene supplement was withdrawn in order to determine how long the animal could maintain itself on the amount of carotene that had been given (9.74 mg.). This animal succumbed 3 weeks after cessation of the carotene medication. Although there were a few necrotic areas on the liver that were indicative of infection, the evidence pointed to an old infection rather than an active one. Since there was incrustation of the eyes and the cornea of one eye was opaque, the animal was considered to have died in a state of vitamin A deficiency.

STORAGE OF VITAMIN A FROM DIETARY CAROTENE AND VITAMIN A

A group of male guinea pigs from the stock colony, weighing 200 to 300 gm. each, were weaned to diets A and B (table 1). One pair of ani-

mals on diet B received the supplement of lemon juice only, while the other pair received in addition to lemon juice the carotene-free grass concentrate. When the first symptoms of vitamin A deficiency appeared, one guinea pig in each pair received vitamin A, the other carotene. After the deficiency symptoms had cleared up, the vitamin A and carotene feeding was discontinued to see how long the guinea pig could maintain itself on the stores built up from the amount of vitamin or provitamin given. As soon as the symptoms reappeared the animals were again fed the vitamin or the provitamin, then sacrificed after a period and the livers analyzed. The guinea pigs that received 10 mg. carotene became depleted on the average in 21 days when the supplement was discontinued, whereas those that received 3 mg. of vitamin

TABLE 1

Repeated depletion of male guinea pigs followed by the feeding of vitamin A or carotene.

BASAL DIET	GUINEA PIG NUMBER	TOTAL SUPPLEMENT IN 20 DAYS AFTER FIRST DEPLETION	PERIOD REQUIRED FOR SECOND DEPLETION	SUPPLEMENT FED AFTER SECOND DEPLETION	VITAMIN A IN LIVER
			days		*γ.*
A	223 [1]	Carotene, 10 mg.	20	Carotene, 2 mg. in 4 days	0
B	229 [1]	Carotene, 10 mg.	23	Carotene, 11.5 mg. in 23 days	trace
B	222	Carotene, 10 mg.	20	Carotene, 11.5 mg. in 23 days	0
A	230 [1]	Vitamin A, 3 mg.	32	Died of hemorrhage, no supplement	0
B	220 [1]	Vitamin A, 3 mg.	32	Vitamin A, 1.9 mg. in 13 days	39
B	219	Vitamin A, 3 mg.	27	Vitamin A, 3.6 mg. in 24 days	213

[1] Received carotene-free dried-grass concentrate equivalent to 1 gm. of dried grass weekly.

A were depleted in 31 days. All the guinea pigs required 20 days for depletion when they were first placed on the diet. This was after the suckling period during which they had eaten the stock diet supplemented with orange juice, as well as the mother's milk, that is, a diet in which the source of vitamin A was chiefly carotene.

In general, no vitamin store was detectable in the carotene-fed animals, yet in spite of this, it took 20 days to deplete these animals and 30 days for the animals that received vitamin A. Judging by the liver values obtained after the second period of supplementary feeding, one must assume that the animals fed vitamin A had some vitamin A stored in the liver which they could draw upon during depletion.

Apparently carotene in amounts ranging from 2 mg. in 4 days to 11.5 mg. in 23 days was inadequate for production of significant stores of vitamin A, but vitamin A, 1.9 to 3.6 mg. in 13 or 24 days allowed the formation of such stores.

The possibility remained that the carotene itself might be stored to some extent in the liver. A depletion period of only 20 or 30 days, however, is what one would expect to find with the vitamins that the animal does not store but requires on a daily intake basis, for example, the water-soluble vitamins. This period is comparable with that required for the depletion of ascorbic acid, which is not considered to be one of the vitamins effectively stored. Even the depletion period for those animals that received the vitamin and presumably stored it was relatively short.

The site of vitamin A storage

Likewise it seemed that there might also exist small undetected stores of liver vitamin A in the carotene-fed animals, or that other storage sites exist. In order to test the latter hypothesis five young animals were used. Three of these had been fed on fresh green grass ad libitum, one had received the distilled vitamin A and one had received the carotene concentrate for several weeks preceding sacrifice. The whole carcasses of the latter two animals, exclusive of the liver and the blood serum, were digested with KOH and the unsaponifiable fraction of the oil tested with the Carr-Price reagent. The liver and serum were examined separately. No vitamin A was found in the carcasses or in the liver of the carotene-fed animal but 150 μg. in the liver of the vitamin A fed guinea pig and 40 μg. per 100 ml. serum. The carotene-fed animal had 22 μg. per 100 ml. serum.

The three grass-fed guinea pig specimens were pooled and kidneys, lungs, adrenals, hearts, washed gastro-intestinal tracts, testes, as well as livers, sera and remaining carcasses were examined separately. No vitamin A was found except in sera, livers and a trace in the kidneys. The totals were 33 μg. per 100 ml. sera, 1850 μg. in the three livers, and 5 μg. in the combined kidneys.

Rate of depletion of vitamin A stores

The range and rate of depletion in apparently healthy young animals were investigated. Sixteen guinea pigs purchased from various commercial growers were maintained on a diet of alfalfa hay and rolled barley with carrots fed twice weekly for several weeks. The guinea pigs were then sacrificed and the livers analyzed for vitamin A. Since these animals had been purchased from various growers the early dietary history varied. The diet generally used by growers consists of oat hay, rolled barley, alfalfa (pellets or hay), greens or green vegetable tops,

and sometimes carrots. Only one guinea pig out of sixteen had a measurable amount of vitamin A in its liver; one other showed a trace. This seemed to indicate even poorer utilization of carotene as a source of vitamin A for storage than was found by Chevallier and Choron ('35, '36). Clausen and McCoord ('34) reported that 1 mg. of carotene daily for 2 weeks did not increase the vitamin A content of the guinea pig's liver.

Of the group of female guinea pigs used for depletion studies some weighed 250 to 300 gm., and some that were older weighed 340 to 480 gm. Five were placed on diet G. Four of the animals maintained on diet G did not receive vitamin A and manifested symptoms of vitamin A deficiency. They died shortly after the appearance of the deficiency-symptoms and no vitamin A was found in the livers. The fifth animal, 226, was given 0.5 mg. carotene in 0.25 ml. oil daily a week after deficiency signs appeared. At the end of 31 days the amount of carotene given was doubled. This guinea pig received a total of 53.5 mg. carotene in 69 days after having been depleted to the deficient stage (fig. 1), but had a store of only 50 μg. vitamin A in the liver. Four female guinea pigs, littermates of the animals placed on diet G, were placed on stock diet. After the growth curve was established for the four animals on the stock diet, which contained 0.58 mg. carotene per 100 gm., they were fed different forms of carotene for 38 days to determine the extent of the vitamin storage. Two were given 1.0 mg. carotene in dried grass (cerophyl) daily, plus 1.0 ml. of cottonseed oil; the other two received the same amount of carotene, 1.0 mg. daily, in the form of 1.0 ml. of a carotene oil concentrate. For guinea pigs weighing 500 gm., this amount was equivalent to 2.0 mg. per kilogram of body weight daily, which is sixteen times the amount reported as necessary for significant storage in the other mammals that have been studied (Guilbert, Howell and Hart, '40). Chemical analysis of the livers revealed a small amount of vitamin A in all the livers. The values ranged from a trace to 0.8 μg. per gram and the maximum total storage was only 23 μg. for the entire liver. This is a low level of storage considering the amount ingested.

An experiment was carried out to test the speed with which the vitamin A stored in the guinea pig's liver disappeared when the dietary intake was low. Twenty-eight guinea pigs bedded on oat hay and raised on a diet of fresh grass and rolled barley were maintained on the stock diet which provided 0.1 to 0.2 mg. carotene a day. These animals were sacrificed at various intervals and the livers and sera analyzed for vitamin A (table 2). The loss of vitamin A from the liver was rela-

tively rapid. In four of the six animals sacrificed after 30 days on the stock diet, the amount of vitamin A present was so low as to be barely detectable. Of the seven animals sacrificed on the thirty-fourth day the livers of three were devoid of the vitamin. The stores remaining at the end of the 36-day period were low. Fluctuations of serum vitamin occurred with a tendency toward a decrease in the females. There were no signs of vitamin A deficiency in any animal, and on this stock diet of low carotene content the young guinea pigs grew satisfactorily.

TABLE 2

Guinea pigs on low-carotene stock diet showing rate of depletion of liver stores of vitamin A which had been built up by feeding fresh grass.

	MALES				FEMALES			
PERIOD ON STOCK DIET	Number of animals	Weight when sacrificed	Average serum vitamin A	Average total liver vitamin A	Number of animals	Weight when sacrificed	Average serum vitamin A	Average total liver vitamin A
days		*gm.*	*µg./100 ml.*	*µg.*		*gm.*	*µg./100 ml.*	*µg.*
1	3	324–333	33	584	..	....	..	..
3	2	360–384	33	466	1	315	33	156
15	3	434–455	20	222	..	....	..	..
30	5	455–582	26	67	1	500	28	0
34	6	500–580	23	26	1	500	17	0
36	4	482–557	33	31	2	417 359	12	9

Vitamin A and carotene during reproduction

Although growth may be maintained on the low carotene stock diet even though storage is negligible, it was not known whether reproduction was affected by these circumstances. Reproduction was chosen as representing a period of physiological stress during which any advantage inherent in the possession of large reserves of vitamin A might be demonstrated. In its natural environment the guinea pig consumes large amounts of green feed providing a super-abundance of carotene. In consequence, the mechanism of forming and storing vitamin A may be wasteful, and this may operate to the disadvantage of the animal on carotene-low diets.

Good reproduction had been obtained in the stock colony on the somewhat low intake of carotene provided by the stock diet without additional vitamin A. In spite of this the incidence of abortion was high in the guinea pigs observed during this study on the low-carotene stock diet as well as on diet G. Abortions in guinea pigs due to vitamin A

deficiency have been reported by Manzi ('35). Hart and Guilbert ('41) found cattle to be similarly affected. Forty female guinea pigs were used in an attempt to study (a) the adequacy of carotene as compared with the vitamin in meeting the requirement during reproduction and (b) the adequacy of the liver stores for meeting this requirement after the feeding of the two forms of the vitamin.

On the supplemented low-carotene stock diet, which provided 0.15 mg. carotene daily, the animals that received an additional 1.0 mg. carotene daily terminated five of nine pregnancies successfully but without a store of vitamin A in the liver. Three of the four pregnancies supported by the addition of 0.1 mg. vitamin A daily were terminated successfully with significant storage of vitamin A in the liver of both mothers and young.

After a preliminary period on stock diet with the males, during which they received 1.0 mg. carotene or 0.15 mg. vitamin A daily twenty-seven mature young female guinea pigs were placed on the vitamin A deficient diet G. All six of the animals on this diet without added vitamin A died within 8 to 31 days. Of the ten animals that received 0.5 mg. carotene per day seven were pregnant, four of which aborted, and none delivered viable young. Only one survived the experimental period and none had measurable amounts of vitamin A in the liver. Of the eleven animals that received 0.15 mg. vitamin A per day five survived. Four were pregnant and of these two aborted. Living young were not delivered although nearly all of the animals had stores of vitamin A in the liver. The results are suggestive of the superior value of vitamin A over carotene for reproduction but further work must be done before this can be considered established.

SUMMARY

Simplified diets were devised, which when supplemented with lemon juice, ascorbic acid, and vitamin A or carotene were adequate for the growth of guinea pigs. These diets were used to produce vitamin A deficiency when vitamin A and carotene were omitted.

The symptoms of vitamin A deficiency observed in the guinea pig were similar to those described in other species. These were typical xerophthalmia, weight loss, and death within a few days after the eye symptoms developed, usually 3 to 4 weeks after the young animals were placed on the deficient diet.

Storage of vitamin A appeared to be restricted to the liver except for a trace in the kidneys. The liver stores were small except when the preformed vitamin was fed. Vitamin A appeared to be more than six

times as effective for this purpose as was carotene. Daily amounts of 1.0 mg. carotene per kilogram for 38 days in depleted animals produced only a trace of liver vitamin A, whereas 2.0 mg. per kilogram daily for the same period resulted in storage that was just significant. The loss of these stores of the vitamin was rapid on lowered intake, 90% disappearing in 30 days.

LITERATURE CITED

BOOCK, E., AND J. TREVAN 1922 The food value of mangolds and the effects of deficiency of vitamin A on guinea pigs. Biochem. J., vol. 16, p. 780.

CHEVALLIER, A., AND Y. CHORON 1935 Recherches sur la presence de la vitamine A et de la substance A' dans le tissu hepatique. C. R. Soc. Biol., vol. 118, p. 159.

———— 1936 Sur la mise en reserve de la vitamine A et de la substance A' dans le tissu hepatique. C. R. Soc. Biol., vol. 121, p. 1015.

CLAUSEN, S. W., AND A. B. MCCOORD 1934 The distribution of vitamin A in the tissues of the rat and guinea pig. J. Biol. Chem., vol. 105, Proc. XV — (XVI).

DAVIES, A. W. 1933 Colorimetric determination of vitamin A by the alkali digestion method. Biochem. J., vol. 27, p. 1770.

GUILBERT, H. R., C. E. HOWELL AND G. H. HART 1940 Minimum vitamin A and carotene requirements of mammalian species. J. Nutrition, vol. 19, p. 91.

HART, G. H., AND H. R. GUILBERT 1941 The relation of brucella abortus infection to the vitamin A content of fetal liver. Am. J. Vet. Res., vol. 2, p. 390.

HETLER, R. A. 1934 The development of xerophthalmia and keratinization of epithelial tissue on withdrawal of vitamin A from the diet of the monkey (Macacus rhesus), guinea pig, rabbit and adult albino rat. J. Nutrition, vol. 8, p. 75.

HUBBELL, R. B., L. B. MENDEL AND A. J. WAKEMAN 1937 A new salt mixture for use in experimental diets. J. Nutrition, vol. 14, p. 273.

KOEHN, C. J., AND W. C. SHERMAN 1940 The determination of vitamin A and carotene with the photoelectric colorimeter. J. Biol. Chem., vol. 132, p. 527.

MANZI, D. L. 1925 Valore del carotene nella carenza di vitamin A durante lo stato gravidico. Arch. Ostet. Ginecol., vol. 42, p. 77.

WOLBACH, S. B., AND P. R. HOWE 1928 Vitamin A deficiency in the guinea pig. Arch. Path. and Lab. Med., vol. 5, p. 239.

WOOLLEY, D. W. 1942 Some new dietary essentials required by guinea pigs. J. Biol. Chem., vol. 143, p. 679.

YUDKIN, S. 1941 Estimation of vitamin A and carotene in human blood. Biochem. J., vol. 35, p. 551.

NUTRITIONAL STUDIES ON MILK FAT

I. THE GROWTH OF YOUNG RATS FED MILK FAT AND CERTAIN SYNTHETIC GLYCERIDES AS SUPPLEMENTS TO A FAT-FREE DIET

J. L. HENDERSON, E. L. JACK, SAMUEL LEPKOVSKY AND DELLA F. REID
Division of Dairy Industry, University of California, Davis and Division of Poultry Husbandry, University of California, Berkeley

TWO FIGURES

(Received for publication May 9, 1945)

Until lately, the study of the nutritional value of fats has been complicated by the necessity of using basal diets that contain appreciable quantities of fat supplied in vitamin concentrates. Now, however, one can prepare an adequate basal diet that is essentially fat-free. Such a diet has been compounded and used to compare the growth of rats fed milk fat with the growth of those receiving certain synthetic glycerides as supplements to the basal diet.

EXPERIMENTAL

Test diets

The composition of the fat-free diet (diet 1) is given in table 1. The diets containing fat were made up to contain 20% fat, 30% casein, 46% glucose, 4% salt mixture, and the same vitamin supplements as the fat-free diet. The fats used were trilaurin (diet 2), triolein (diet 3), equal parts trilaurin and triolein (diet 4), and milk fat (diet 5).

Triolein was used because it represents the most widely distributed unsaturated fatty acid. Trilaurin was chosen as a saturated fat that melts near body temperature.

The fat-free diet (no. 1), in preliminary trials, gave satisfactory growth when fed to young rats. The only sources of lipids in this diet besides the residual fat in the casein, are the linoleic acid and the ethyl laurate; the latter was used to carry the fat-soluble vitamins, which were dissolved in an amount of ethyl laurate such that about 0.07 ml. per week supplied the required amount to each rat. To ensure consumption of these vitamins and the linoleic acid, they were fed separate from the rest of the diet. The casein was extracted with alcohol

and ether to a lipid content of 0.2%. The original diet was somewhat dusty and this was corrected by substituting 1% glycerol for 1% glucose.

The milk fat was obtained from the milk of a large number of cows; the method of collection has been described elsewhere (Henderson and Jack, '44). The fatty-acid composition, determined in detail (Jack and Henderson, '45) by ester-fractionation and analysis, is shown in table 2.

TABLE 1

Composition of the fat-free diet (diet 1).

Basic mixture		*Other vitamin supplements fed per kilo of basic mixture*	
Labco casein, alcohol and ether extracted	24%	Thiamine [1]	10 mg.
Dextrose	72%	Riboflavin [2]	10 mg.
Sure's salt mixture [1]	4%	Pyridoxine [3]	10 mg.
		Pantothenic acid [2] (as Ca-pantothenate)	40 mg.
Vitamin supplements		Nicotinic acid	0.1 gm.
Linoleic acid, prepared by fractional crystallization in acetone (−60°C.) of fatty acids of corn oil	25 mg./rat/day	Inositol	1.0 gm.
α-Tocopherol (Eastman)	2 mg./rat/wk.	Para-aminobenzoic acid	0.6 gm.
Vitamin A alcohol (Eastman)	30 μg./rat/wk.	Choline	500 mg.
Vitamin D_3 [2]	1 μg./rat/wk.	Biotin [4]	25 μg.
Vitamin K (1-acetoxy-2 methyl-4-naphthyl sodium phosphate)	2 μg./rat/wk.		

[1] Sure, B., J. Nutrition, vol. 22, p. 499, 1941.
[2] Furnished by courtesy of E. I. du Pont de Nemours and Co., New Brunswick, N. J.
[3] Supplied by courtesy of Merck and Co., Rahway, N. J.
[4] Biotin solution (free acid), S.M.A. Corp., Chagrin Falls, Ohio.

TABLE 2

Fatty-acid composition of milk fat.
(Iodine no. (Hanus) 32.42; saponification no. 230.0; nonsaponifiable matter 0.28).

LENGTH OF CARBON CHAIN	WEIGHT	MOL	LENGTH OF CARBON CHAIN	WEIGHT	MOL
	%	%		%	%
Saturated			Unsaturated		
C_4	3.52	9.2	C_{10}	0.25	0.3
C_6	1.40	2.8	C_{12}	0.15	0.2
C_8	1.68	2.7	C_{14}	1.48	1.5
C_{10}	2.67	3.5	C_{16}	5.69	5.2
C_{12}	4.54	5.2	C_{17}	18.69	15.3
C_{14}	11.67	14.8	C_{20}	0.98	0.7
C_{16}	30.05	27.2	Linoleic	2.12	1.7
C_{18}	10.45	8.5			
C_{20}	1.68	1.2			

Oleic acid for the triolein was prepared from olive oil by the fractional-crystallization method of Brown and Shinowara ('37). The lauric acid for the trilaurin was obtained by ester fractionation of coconut oil. To prepare the triglycerides, direct esterification of the acids with glycerol under bubbling CO_2 was the method used. The esterified products were subsequently washed with 70% alcohol.

The test diets were mixed in small quantities and refrigerated to avoid deterioration.

Feeding trials

Two trials were made using young rats from the stock colony divided into groups of four each, two males and two females. They were maintained in individual cages and fed the diets ad libitum. Over-all feed consumption was measured. In one trial (no. 1) the mothers and young were placed on the fat-free diet when the young were 14 days old. The young rats were weaned at 21 days and placed on the test diets at an average weight of about 48 gm. In the other trial (no. 2) the rats were allowed to grow to about 85 gm. before weaning; they were then placed on the fat-free diet for 1 week before being given the test diets.

DISCUSSION

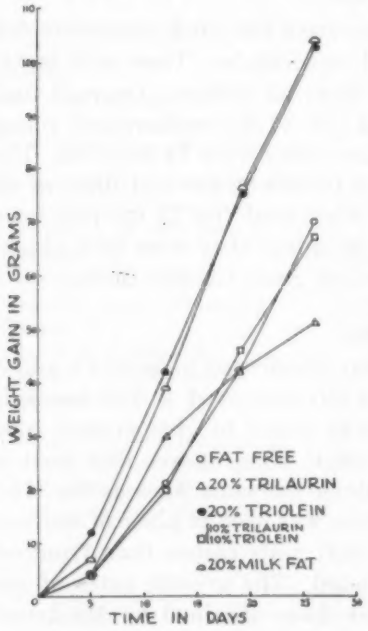
The results of the two feeding trials are illustrated in figures 1 and 2. Mackenzie, Mackenzie and McCollum ('39) prepared a diet lower in fat than any previously reported. It was found to give growth comparable with that made on their stock diet. The fat-free diet used in the present experiments was planned along the same lines as the Mackenzie-McCollum study except that glucose was used in place of sucrose, the B vitamins were provided as pure materials rather than from extracted yeast, and no cystine was included. The growth rates of our rats on fat-free diets closely resembled those obtained by Mackenzie et al. ('39).

The difference in growth rates between the two experiments here reported can be explained by the difference in starting weights. The smaller rats grow more rapidly than the larger ones; but if the curves are superimposed so that the ordinates coincide at the same body weight, they form segments of the same general curve.

It is difficult to ascribe any significance to the fat-depletion period, either of the mother before weaning or of the young rats after weaning. In both trials, rats receiving the milk fat and the triolein showed faster growth rates than those receiving the other diets. There was no significant difference between the milk fat and the triolein diets.

Trilaurin restricted growth as has been reported earlier in the literature (Evans and Lepkovsky, '32 a, '32 b; Cox, '33) and the effect was more pronounced in the younger rats. When trilaurin was mixed with triolein so that each provided 10% of the diet, this diet was less restrictive of growth than when the diet contained 20% trilaurin.

Feed consumption was almost identical for all lots, except that those on the trilaurin ate slightly less feed than the others. This difference, however, being only about 5% less feed per rat for the entire feeding period, was too small to account for the difference in growth.



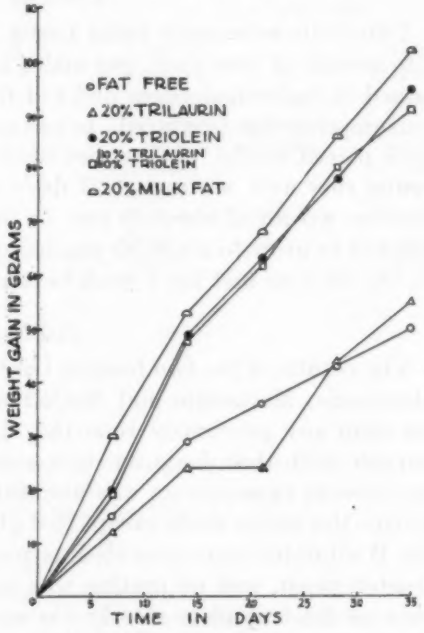


Fig. 1 Weight gain of rats in feeding trial 1.
Fig. 2 Weight gain of rats in feeding trial 2.

SUMMARY AND CONCLUSION

1. A fat-free diet is described in which all the components are pure compounds with the exception of casein extracted with alcohol and ether.

2. This diet was found to be satisfactory for use in the fat-nutritional experiments.

3. The rats on the milk-fat diet and on the triolein diet grew faster than those on the other diets. There was no significant difference be-

tween the milk-fat diet and the triolein diet with respect to their effect on growth.

4. Trilaurin, when fed at the level of 20%, enabled the rats to grow at about the same rate or slightly slower than the rats on a fat-free diet.

5. When 10% each of trilaurin and triolein were fed, the growth of the rats was intermediate between those receiving trilaurin and those receiving triolein.

6. The growth-promoting effects of the different diets could not be attributed to differences in feed consumption.

LITERATURE CITED

BROWN, J. B., AND G. Y. SHINOWARA 1937 Studies on the chemistry of the fatty acids. II. The preparation of pure oleic acid by a simplified method. Am. Chem. Soc. J., vol. 59, p. 6.

COX, W. M. 1933 The nutritive value of pure fatty acid esters. J. Biol. Chem., vol. 103, p. 777.

EVANS, H. M., AND S. LEPKOVSKY 1932 a Vital need of the body for certain unsaturated fatty acids. II. Experiments with high fat diets in which saturated fatty acids furnish the sole source of energy. J. Biol. Chem., vol. 96, p. 157.

———— 1932 b The sparing action of fat on vitamin B. III. The role played by glycerides of single fatty acids. J. Biol. Chem., vol. 96, p. 179.

HENDERSON, J. L., AND E. L. JACK 1944 The fractionation of milk fat from a solvent at low temperatures. Oil and Soap, vol. 23, p. 90.

JACK, E. L., AND J. L. HENDERSON 1945 The fatty-acid composition of glyceride fractions separated from milk fat. J. Dairy Sci., vol. 28, p. 65.

MACKENZIE, C. G., J. B. MACKENZIE AND E. V. MCCOLLOM 1939 Growth and reproduction on a low fat diet. Biochem. J., vol. 33, p. 935.

NUTRITIONAL STUDIES ON MILK FAT

II. THE GROWTH OF YOUNG RATS FED GLYCERIDE FRACTIONS SEPARATED FROM MILK FAT

E. L. JACK, J. L. HENDERSON, DELLA F. REID AND SAMUEL LEPKOVSKY

Division of Dairy Industry, University of California, Davis and Division of Poultry Husbandry, University of California, Berkeley

ONE FIGURE

(Received for publication May 9, 1945)

The opinion is current in many places that food fats of either animal or vegetable origin, when properly supplemented, are practically equivalent in nutritional value. The Council on Foods and Nutrition of the American Medical Association ('42) drew the following conclusion: "At present there is no scientific evidence to show that the use of fortified oleomargarine in the average adult diet would lead to nutritional difficulties. A similar conclusion is probably justified in the case of growing children." This statement has been taken by many persons to imply that oleomargarine is nutritionally equal to milk fat. The Council, however, further points out: "The nutritional factors have not all been identified and butter contains numerous additional fatty acids of unknown nutritional significance."

Deuel and associates ('44) were able to find no difference in the growth rate of rats fed diets supplemented with butter, corn oil, cottonseed oil, margarine, olive oil, peanut oil, and soybean oil. The fats were fed at approximately a 30% level. On the other hand Gullickson, Fountaine and Fitch ('42) found that young calves did poorly and often died when their sole source of fat was vegetable oils, but that they were healthy and thrifty on milk fat, lard, or tallow. Elvehjem, Hart, and their co-workers ('40, '40, '41) showed that milk fat gave better growth when fed to weanling rats than did corn oil, coconut oil, cottonseed oil, and soybean oil. The superiority of milk fat was most pronounced when lactose was the carbohydrate used (Boutwell et al., '43). The corn oil was superior when dextrin, sucrose, glucose, or starch was used. According to these results and others reported in the literature, there is no clear-cut evidence that one fat is nutritionally superior to another.

The present paper reports the results obtained when glyceride fractions from a natural fat (milk fat) were fed to weanling rats as supplements to a fat-free diet. This approach to the problem was chosen because if any portions of a natural fat (whose composition is known) can be shown to differ nutritionally from other portions, then a basis will be established for determining the inherent nutritional value of a fat.

EXPERIMENTAL

Fat fractions

Milk-fat fractions were prepared by precipitation from a solvent at progressively lower temperatures, as follows: — 7°, — 13°, — 23°, — 53°C., and the filtrate remaining at — 53°C. The details of the preparation of these fractions have been described elsewhere (Henderson and Jack, '44). In physical appearance these fractions ranged from a dry white powder to a reddish oil; the melting point ranged from 53° to — 10°C., and the iodine numbers from 8.2 to 56. The detailed fatty-acid composition of the fractions and of the original milk fat is shown in table 1 (Jack and Henderson, '45).

TABLE 1

Fatty acid composition of milk fat and of milk-fat fractions.

LENGTH OF CARBON CHAIN	MOL PER CENT					
	— 7 ppt.	— 13 ppt.	— 23 ppt.	— 53 ppt.	— 53 filt.	Milk fat
Saturated						
C_4	2.5	4.6	8.7	7.9	9.3	9.2
C_6	..	2.9	4.0	5.8	6.9	2.8
C_8	..	6.6	1.3	1.7	2.4	2.7
C_{10}	1.2	4.9	4.0	5.5	4.5	3.5
C_{12}	4.5	3.5	4.2	4.7	3.7	5.2
C_{14}	16.6	11.3	14.6	16.6	7.4	14.8
C_{16}	42.1	36.2	38.1	24.0	14.0	27.2
C_{18}	21.1	9.3	5.2	8.5	6.8	8.5
C_{20}	3.8	3.1	1.0	1.4	1.4	1.2
C_{22}	0.8	..	..	..	..	..
Unsaturated						
C_{10}	..	0.2	0.1	0.3	1.4	0.3
C_{12}	..	0.2	0.1	0.2	0.4	0.2
C_{14}	0.1	0.9	1.2	1.4	1.8	1.5
C_{16}	0.4	3.9	4.5	2.7	4.6	5.2
C_{18}	6.1	10.6	10.7	17.2	30.0	15.3
C_{20}	0.8	1.3	1.9	1.8	0.8	0.7
Linoleic	..	0.5	0.4	0.5	6.0	1.7

Test diets

Table 2 shows the compositions of the diets.

TABLE 2

Composition of test diets.[1]

DIET NO.	FAT SOURCE AT 20% LEVEL	CASEIN	GLUCOSE	SUBE'S SALT MIX
		%	%	%
1	Fat-free	24	72	4
2	Milk fat (solvent treated)	30	46	4
3	Synthetic [2] milk fat	30	46	4
4	— 7 ppt.	30	46	4
5	— 13 ppt.	30	46	4
6	— 23 ppt.	30	46	4
7	— 53 ppt.	30	46	4
8	— 53 filt.	30	46	4
9	Composite [3] milk fat	30	46	4
10	Milk fat (not solvent treated)	30	46	4

[1] The diets were supplemented by vitamins, as described previously. See table 1 in paper entitled "Nutritional Studies on Milk Fat. I." by Henderson and Jack, vol. 30, p. 170.

[2] Fatty acids of milk fat reesterified with glycerol.

[3] Combined increments of the different fractions.

Feeding technic

The selection of the rats, the numbers used, and the methods of feeding were the same as those previously described (Henderson et al., '45).

RESULTS

Two feeding trials were made, and the results of trial 2 are shown in figure 1 as being representative. The findings were practically identical in the two trials. Trial 2 was chosen for presentation because it included diets 2 and 3 which were not in the first trial. Diet 2 contained milk fat which had been dissolved in solvent followed by solvent removal in the same manner as the fat fractions. The fat in diet 3 was synthesized from the component fatty acids of milk fat.

DISCUSSION

The experimental data illustrated in figure 1 show the diet containing untreated milk fat to be superior to the others. The remainder of

the diets, all of which contained fat which had been dissolved in solvent at some stage in its preparation, may be divided essentially into three groups on the basis of their growth-promoting ability: the diet containing the — 53° filtrate was markedly superior to the rest; the diets containing the — 7° ppt. and the fat-free diet were the poorest; the others, while showing some variation within themselves, were all sufficiently in the same range to constitute a single group. The authors are not prepared to attempt an explanation of the effect of the solvent.

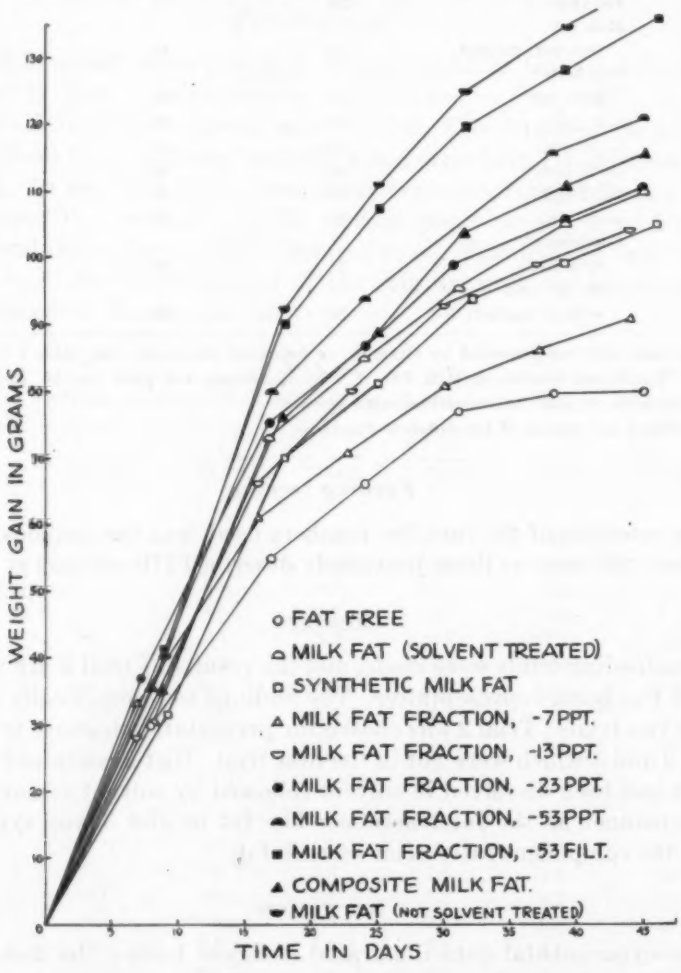

WEIGHT GAIN IN GRAMS
TIME IN DAYS
FAT FREE
MILK FAT (SOLVENT TREATED)
SYNTHETIC MILK FAT
MILK FAT FRACTION, -7 PPT.
MILK FAT FRACTION, -13 PPT.
MILK FAT FRACTION, -23 PPT.
MILK FAT FRACTION, -53 PPT
MILK FAT FRACTION, -53 FILT.
COMPOSITE MILK FAT.
MILK FAT (NOT SOLVENT TREATED)

Fig. 1 Weight gain of rats on diets containing milk-fat derivatives.

The diets containing the fat fractions, even though the two extreme fractions are outstanding, all ranged themselves in the order of the temperature of precipitation. These differences cannot be ascribed to differences in assimilation resulting from differences in melting point (Henderson and Jack, '44), since all except the — 7° ppt. melt at temperatures at which assimilation is known to be excellent, and since even 53°C., the melting point of the — 7° ppt., was not high enough to interfere seriously with assimilation (Boutwell et al., '43).

Reference to the composition of the fat fractions (table 1) suggests that the oleic acid content or possibly the total unsaturation might be a contributing factor since this is the only characteristic that varies in the same manner as the growth-promoting ability. Conceivably, the marked superiority of the — 53° filtrate might also result from the fact that this fraction is highly unsaturated and contains most of the nonsaponifiable matter of milk fat. This possibility, however, does not appear probable, since preliminary trials have shown the fat-free diet to be vitamin-sufficient; it is believed that the principal role of these factors is to supply vitamin activity.

The results presented here suggest conclusions different from those of the Wisconsin workers (Schantz et al., '40), wherein they reported that the saturated portion of milk fat provided better growth than the other portions. Their saturated portion compared in degree of unsaturation with our — 7° ppt., which was inferior to the other fractions. The results here were on the natural glycerides, whereas the Wisconsin results were based on glycerides synthesized from "solid" fatty acids separated from milk fat. The results obtained by the Wisconsin feeding technic could be interpreted as showing the value of saturated fatty acids of milk as supplements to corn oil, since the fatty-acid fractions fed had been mixed with and replaced a certain amount of corn oil. Possibly the differences were due to the synergistic action of the two fats, or fat-soluble constituents, rather than to an inherent property of either. In the results here reported, the differences are believed to be true representations of the growth-promoting properties of milk-fat fractions, inasmuch as they constituted the sole source of fat.

Essentially similar results were obtained with the natural milk fat, treated with the same solvent as the fractions; the synthetic milk fat, prepared by esterification of the milk-fat fatty acids with glycerol; and the composite milk fat, obtained by mixing the proper increments of the milk-fat fractions. The results fell about midway in the intermediate group. The synthetic milk fat does not contain the nonsaponifiable matter of milk fat, which is present in the other two.

Burr and Barnes ('43) have commented on the large differences that have been reported on the comparative nutritional value of different lipids. According to them, the outstanding feature of these experiments is the irregularity of results, which they ascribe either to the method of feeding or to the unrecognized effect of rancidity on the rest of the diet. We do not believe that either of these two factors affected our findings, since the experiments were repeated after 5 months with the same results, using fat from the same source.

SUMMARY AND CONCLUSION

1. Five glyceride fractions separated from milk fat by precipitation from a solvent were fed to weanling rats, and the gain in weight was measured.

2. The growth responses place the fractions in three groups. The — 53° filtrate was superior to all other diets containing solvent-treated fat; the — 7° ppt. and the fat-free were the poorest; the others formed an intermediate group.

3. The diet containing natural milk fat which had not been solvent-treated gave greater growth than any other diet. The diets containing the solvent-treated milk fats (natural, synthetic and composite) fell in the intermediate group with no significant difference between them.

4. The fatty acid composition of the fats suggests that either the oleic acid content or the total unsaturation might be a factor contributing to differences in growth.

LITERATURE CITED

BOUTWELL, R. K., R. P. GEYER, C. A. ELVEHJEM AND E. B. HART 1941 The effect of hydrogenation on the nutritive value of the fatty acid fractions of butter fat and of certain vegetable oils. J. Dairy Sci., vol. 24, p. 1027.

———— 1943 Further studies on the growth promoting value of butter fat. J. Dairy Sci., vol. 26, p. 429.

BURR, G. O., AND R. H. BARNES 1943 The non-caloric value of dietary fats. Physiol. Rev., vol. 23, p. 256.

COUNCIL ON FOODS AND NUTRITION OF THE AMERICAN MEDICAL ASSOCIATION 1942 The comparative nutritional value of butter and oleomargarine. J. Am. Med. Assn., vol. 119, p. 1425.

DEUEL, H. J., JR., E. MOVITT, L. F. HALLMAN AND F. MATTSON, WITH TECHNICAL ASSISTANCE OF E. BROWN 1944 Studies on the comparative nutritive value of fats. I. Growth rate and efficiency of conversion of various diets to tissue. J. Nutrition, vol. 27, p. 107.

GULLICKSON, T. W., F. C. FOUNTAINE AND J. B. FITCH 1942 Various oils and fats as substitutes for butterfat in the ration of young calves. J. Dairy Sci., vol. 25, p. 117.

HENDERSON, J. L., AND E. L. JACK 1944 The fractionation of milk fat from a solvent at low temperatures. Oil and Soap, vol. 21, p. 90.

HENDERSON, J. L., E. L. JACK, S. LEPKOVSKY AND D. F. REID 1945 Nutritional studies on milk fat. I. The growth of young rats fed milk fat and certain synthetic glycerides as supplements to a fat-free diet. J. Nutrition, vol. 30, p. 169.

JACK, E. L., AND J. L. HENDERSON 1945 The fatty-acid composition of glyceride fractions separated from milk fat. J. Dairy Sci., vol. 28, p. 65.

SCHANTZ, E. J., R. K. BOUTWELL, C. A. ELVEHJEM AND E. B. HART 1940 The nutritive value of the fatty acid fractions of butter fat. J. Dairy Sci., vol. 23, p. 1205.

SCHANTZ, E. J., C. A. ELVEHJEM AND E. B. HART 1940 The comparative nutritive value of butter fat and certain vegetable oils. J. Dairy Sci., vol. 23, p. 181.

ENZYMATIC RELATIONSHIPS IN THE UTILIZATION OF SOYBEAN OIL MEAL PHOSPHORUS BY THE RAT [1]

ROBERT R. SPITZER AND PAUL H. PHILLIPS

Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison

TWO FIGURES

(Received for publication May 31, 1945)

INTRODUCTION

In a study of the availability of soybean oil meal phosphorus (Spitzer and Phillips, '45) it was found that 58.0% of the soybean oil meal phosphorus existed in the form of phytin or phytic acid and that this phosphorus was readily available to the rat.

In view of these results an explanation for the utilization of soybean oil meal phosphorus was sought by investigating the role of phytin and phytic acid-splitting enzymes.

A phytic acid-splitting enzyme (phytase) was first found by Suzuki et al. ('06). Adler ('16) studied a malt phytase and found its optimum hydrogen ion concentration at pH 5.4. Horiuchi ('31) found that rice bran contained a phytase with optimum hydrogen ion concentration at pH 4.3.

It has been suggested by Hart et al. ('09), by Pedersen ('41) and by Singsen and Mitchell ('44) that there may be some relationship between the availability of phytic acid phosphorus and the phytase activity of the ration. McCance and Widdowsen ('44) and Mellanby ('44) have discussed the role of phytase in wheat and oats with respect to the difference in rachitogenic effect between the two grains.

The occurrence of phytase in the animal body was reported by McCollum and Hart ('08). These workers found phytase activity in the liver and blood of calves. Plimmer ('13) failed to find the enzyme in

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This study has been supported in part by a grant from the A. E. Staley Manufacturing Company, Decatur, Illinois.

We are indebted to Merck and Co., Rahway, New Jersey, for the synthetic vitamins; to Wilson and Company, Chicago, Illinois, for the 1:20 liver powder; to Abbott Laboratories, North Chicago, Illinois, for halibut liver oil, and to Oscar Mayer Packing Company, Madison, Wisconsin for animal tissues used in these studies.

extracts of the intestines of the dog, sheep, ox and rabbit. Lowe and Steenbock ('36) failed to find phytin-phosphatase activity in the extracts of the mucosa of the small intestine of the chick or the rat. These workers suggested that the hydrolysis occurring in the tract was due to the activity of the intestinal flora or to the phytase in the vegetable part of the ration.

Patwardhan ('37) reported phytase activity in the intestine of the albino rat. This enzyme exerted its optimum activity at pH 7.8 and its action was reported to be accelerated by magnesium ions. Kreiger ('38) reported the existence of a phytin-splitting enzyme in the small intestinal wall of the rat and the chick. Magnesium ions when present at the proper concentration had a stimulating effect on phytase activity. The optimum pH range for this enzyme was between pH 6.8 and pH 7.0. Rapoport et al. ('41) found no phytase activity in the cells or plasma of several species of mammals, but phytase was present in the plasma of lower vertebrates. This enzyme showed optimum activity at a slightly acid pH and was not stimulated by magnesium ions.

The earlier studies by Spitzer and Phillips ('45)[2] which suggested that the presence of an enzyme in soybean oil meal was not required for utilization of soybean oil meal phosphorus by the rat did not rule out the possibility that other ration constituents possessed phytase activity.

The purpose of the present experiment was to determine if the availability of the soybean oil meal phosphorus was due to the action of a ration-borne enzyme, or the presence of phytase in the gastrointestinal tract or in the organs themselves. To this end a method for measuring phytase activity was developed, and the phytase activity of several soybean oil meals, rations, and tissues was measured.

EXPERIMENTAL

Studies were made with 40 gm. weanling rats fed a semi-synthetic basal ration which was low in phosphorus to determine if the availability of soybean oil meal phosphorus was due to the presence of phytase in the ration. The basal ration differed from the one used in

[2] The ration used in these studies was similar in composition to the low-P-fibrin ration used by Jones (J. Nut. 15, 269, 1942) in that it contained fibrin as the source of protein and a low phosphorus salt mixture. The publications of Jones and associates have amply demonstrated that their low phosphorus-fibrin rations were adequate for growth and bone formation in the rat when phosphorus was added. The growth rates and bone ash values obtained by Spitzer and Phillips, '45 are in very close agreement to those obtained by Jones and Foster (J. Nut. 24, 245, 1942).

earlier studies in one respect; namely, the 1% 1:20 liver powder was omitted because it was a constituent which might possess some phytase activity. This basal ration which will be called the "basal-B" ration consisted of 77.6% sucrose, 14.0% fibrin, 2% low calcium low phosphorus salts, 4% corn oil, 0.1% inositol and 0.3% choline chloride. The other vitamins were fed at the same level and in the same manner that has been reported earlier (Spitzer and Phillips, '45). Vitamin D was fed at a level of 70 U.S.P. units per week.

The "basal-B" ration contained 0.014% phosphorus as determined by the method of Fiske and Subbarow ('25) and the use of an Evelyn photoelectric colorimeter. The phosphorus content of all rations was determined in order to provide data on the level of this element fed throughout the experiment. The calcium content of the rations in all cases was kept as near as possible to 0.6% supplied either as $CaHPO_4$ or $CaCO_3$ or both. The Ca : P ratio varied within the limits of 2.4 : 1 and 2.8 : 1. Inorganic phosphorus was added as $CaHPO_4$. All major substitutions in the "basal-B" ration were made at the expense of the sucrose or fibrin or both.

Rations were stored in the refrigerator to avoid the development of rancidity. Food and distilled water were supplied daily ad libitum. The rats, six per lot, were kept in cages of galvanized wire.

At the end of 5 weeks the animals were sacrificed, the femora removed and bone ash determined by the technique described in earlier experiments (Spitzer and Phillips, '45). The dietary regimen is given in table 1.

RESULTS

Growth data are summarized in figure 1. Inspection of these data shows that when 1.0% 1:20 liver powder was fed with fibrin or soybean oil meal, better growth resulted than when the fibrin or soybean oil meal was fed alone. Heating either the solvent or the expeller type soybean oil meal portion of the ration at 98°C. for 48 hours had no detrimental effect upon growth. When, however, the entire rations were heated at 98°C. for 48 hours, a marked reduction in food consumption occurred which resulted in a retardation of growth rate.

Table 1 summarizes the data on the availability of soybean oil meal phosphorus as measured by bone ash. It is evident that heating the soybean oil meals or the entire ration had no unfavorable effect on the availability of the phosphorus. Neither did the removal of the 1.0% 1:20 liver powder. Slightly higher bone ash values were obtained when 1.0% of 1:20 liver powder was present but this small increase may be

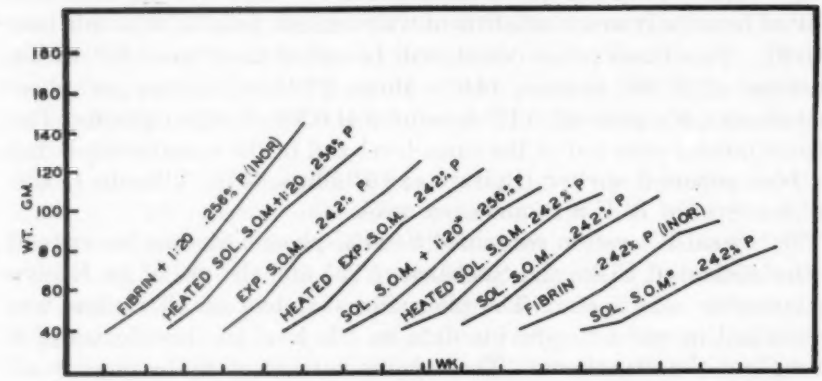


Fig. 1 The effect of heat and the removal of 1.0% liver powder on growth when certain soybean oil meal and fibrin rations were fed. The entire ration was heated for 48 hours at 98°C.

TABLE 1

The effect of heat on the availability of soybean oil meal phosphorus.

LOT NO.	NO. OF RATS	RATION	PER CENT PHOSPHORUS IN RATION	PER CENT CALCIUM IN RATION	AVERAGE PER CENT BONE ASH
1	6	Basal B without fibrin + 34% expeller soybean oil meal (14% protein and 0.228% P)	0.242	0.69	54.2
2	6	Basal B without fibrin + 34% expeller soybean oil meal (heated 48 hours, 98°C., 14% protein and 0.228% P)	0.242	0.69	54.1
3	6	Basal B without fibrin + 34% solvent soybean oil meal (not toasted, 14% protein and 0.228% P)	0.242	0.69	52.0
4	6	Basal B without fibrin + 34% solvent soybean oil meal (heated 48 hours, 98°C., 14% protein and 0.228% P)	0.242	0.69	52.0
5	6	Basal B without fibrin + 34% solvent soybean oil meal (heated 48 hours, 98°C., 14% protein and 0.228% P) + 1% 1: 20 liver powder	0.256	0.69	53.2
6	6	Basal B without fibrin + 34% solvent soybean oil meal (14% protein and 0.228% P). Entire ration heated 48 hours, 98°C.	0.242	0.69	50.5
7	6	Basal B without fibrin + 34% solvent soybean oil meal (14% protein and 0.228% P) + 1% 1: 20 liver powder. Entire ration heated 48 hours, 98°C.	0.256	0.69	51.0
8	6	Basal B + 0.228% inorganic P	0.242	0.60	47.1
9	6	Basal B + 1% 1: 20 liver powder + 0.228% inorganic P	0.256	0.60	48.5

explained on the basis of the phosphorus present in the 1:20 liver powder itself. This slight increase was also observed on the fibrin rations where inorganic phosphorus was employed. It is interesting to observe that although heating the ration caused a depression in growth (fig. 1) and in food consumed, it had very little or no effect on bone ash value.

These results indicate that the utilization of soybean oil meal phosphorus was not dependent upon a ration-borne heat labile phytase.

Since Patwardhan ('37) and Kreiger ('38) had reported phytase activity in the intestine of the rat, it seemed possible that this was the source of the enzyme responsible for the utilization of the soybean oil meal phosphorus. Consequently a method for the measurement of phytase activity was developed for the purpose of ascertaining its distribution in tissues and to check the phytase activity of our rations.

Determination of phytase

Preparation of substrate. Calcium phytate was dissolved in dilute hydrochloric acid and the solution filtered. An equivalent amount of sodium oxalate was then added and the solution was made slightly alkaline. The precipitate of calcium oxalate was removed by filtration. The filtrate containing the sodium phytate was analyzed for inorganic and total phosphorus by the method of Fiske and Subbarow ('25). The results showed the presence of 100 μg. of sodium phytate phosphorus and 10.0 μg. of inorganic phosphorus per milliliter.

Preparation of samples. Albino rats were decapitated, the small intestines were removed, opened, washed carefully in running water, and homogenized by the Potter-Elvehjem technique ('36). Large animal intestines were obtained immediately after the animals were slaughtered and were treated in the manner described above. Thus samples of intestinal walls were prepared for analyses. Samples of intestinal contents were extracted with distilled H_2O and the insoluble material removed by filtration. Plant tissues were prepared by making a water extract of a finely ground sample.

Method of assay. To 2.0 ml. of substrate and 3.0 ml. of buffer, 1.0 ml. of the tissue preparation was added. Inorganic phosphorus was determined immediately before and after a suitable incubation period at 37°C. The increase in inorganic phosphorus represented the phytase activity when other mechanisms for the release of phosphorus were accounted for. The proteins were precipitated by the addition of 3 ml. of 10.0% trichloracetic acid. After standing for 10 minutes the material

was filtered and a suitable aliquot used for the phosphorus determination. The increase in inorganic phosphorus due to the action of other phosphatases and to the breakdown of other phosphorus compounds present in the tissue was controlled by replacing the sodium phytate substrate with distilled water and measuring the liberated phosphorus after incubation. Omission of the tissues from the media resulted in no increase in inorganic phosphorus after an incubation period of 6 hours, which indicated that the substrate could not be hydrolyzed by acid or base alone in the absence of phytase. Thus the increase in inorganic phophorus, due to the action of phytase on the sodium phytate was obtained by difference.

Results of phytase experiments

Preliminary phytase determinations using the above method indicated the presence of the enzyme in the mucosa of the small intestine of the rat. Further analyses were made to study the effect of time, of hydrogen ion concentration, and of magnesium ions on the enzyme's action. Other tissues were also analyzed for the enzyme.

The effect of time on the hydrolysis of sodium phytate by rat intestine. An incubation period of 6 hours was chosen since incubation for this period of time resulted in a readily measurable increase in inorganic phosphorus if phytase was present. Tissues showing no phytase activity after 6 hours also failed to show activity after a 48-hour incubation period.

The effect of hydrogen ion concentration on the hydrolysis of sodium phytate by rat intestine. These results are summarized in figure 2. Potassium acid pthalate buffers were used to give pH 2.2, 3.0, 4.0, 5.2 and 6.0 A sodium acetate buffer was used at pH 7.0 and borate buffers were used at pH 7.8, 9.0 and 10.0. The original media contained approximately 200 μg. of sodium phytate phosphorus. Maximum activity was observed in the region of pH 7.8 where 197 μg. or approximately 98% of the phytate phosphorus was liberated during the 6-hour incubation period.

The influence of magnesium ions on the hydrolysis of sodium phytate by rat intestine. Duplicate determinations were carried out simultaneously to study the effect of magnesium ions on the action of phytase. The media was buffered at pH 7.8 and a 6-hour incubation period was used. The results of the studies are outlined in table 2. Although the addition of a few crystals of $MgSO_4$ to the media resulted in an increase in total phosphorus liberated, it had no stimulating effect on the phytase activity at this pH.

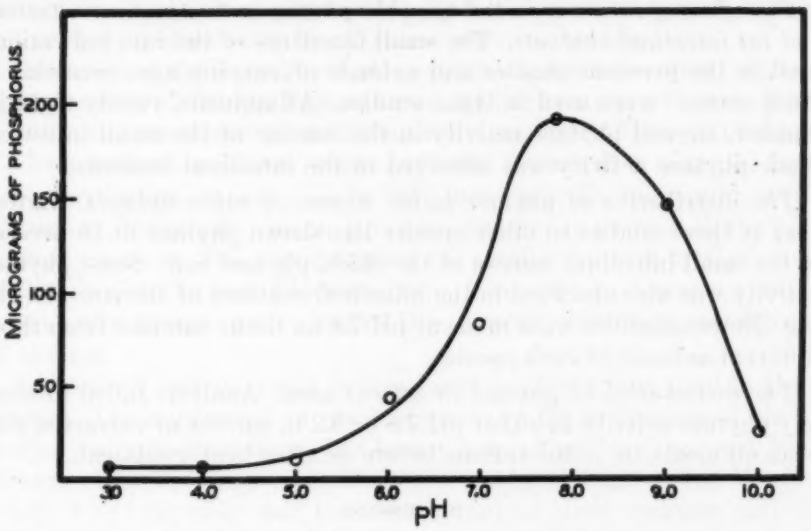


Fig. 2 The effect of hydrogen ion concentration on the hydrolysis of sodium phytate by rat intestine.

TABLE 2

The effect of magnesium ions on the total phosphatase and phytase activity of rat intestine.

	Added $MgSO_4$	Incubation time in hours	Micrograms phosphorus liberated	Micrograms Na phytate phosphorus liberated
Enzyme preparation, no. 1				
With substrate	0	0		
With substrate	0	6	237	
Without substrate	0	0		
Without substrate	0	6	73	164
With substrate	+	0		
With substrate	+	6	246	
Without substrate	+	0		
Without substrate	+	6	103	143
Enzyme preparation, no. 2				
With substrate	0	0		
With substrate	0	6	224	
Without substrate	0	0		
Without substrate	0	6	72	152
With substrate	+	0		
With substrate	+	6	252	
Without substrate	+	0		
Without substrate	+	6	109	143

The effect of ration ingested upon the phytase activity of rat intestine and rat intestinal contents. The small intestines of the rats fed rations used in the previous studies and animals of varying ages receiving a stock ration [3] were used in these studies. All animals, twenty-eight in number, showed phytase activity in the mucosa of the small intestine. Some phytase activity was observed in the intestinal contents.

The distribution of phytase in the tissues of other animals. Extension of these studies to other species has shown phytase to be present in the small intestinal mucosa of the chick, pig and cow. Some phytase activity was also observed in the intestinal contents of the cow and the pig. Determinations were made at pH 7.8 on tissue samples from three different animals of each species.

The distribution of phytase in rations used. Analysis failed to show any phytase activity at either pH 7.8 or 5.2 in solvent or extracted soybean oil meals, or in the rations before or after heat treatment.

DISCUSSION

The crude enzyme preparations used in these studies undoubtedly contained some magnesium ions. Further additions of magnesium ions resulted in an increase in total inorganic phosphorus liberated but had no stimulating effect on the phytase. Kay ('32) reported that an intestinal phosphatase, capable of hydrolyzing monophosphoric esters, was stimulated by magnesium ions. However, the phytase reported by Rapoport et al. ('41) showed its maximum activity at a slightly acid pH and was not stimulated by magnesium ions. Similar to our studies, Rapoport used methods for determining the increase in inorganic acid phosphorus due specifically to phytase. This suggests that the stimulation observed by Patwardhan ('37) and Kreiger ('38) was due to the activation of other phosphatases, as shown by Kay ('32). The question of concentration of magnesium ions should not be overlooked.

It seems quite possible that the enzyme studied here was the same phytase reported by Patwardhan ('37) and possibly similar to that reported by Kreiger ('38). On the other hand the existence of several animal "phytases" is quite possible.

Kreiger and Steenbock ('40) have shown that the utilization of phytin phosphorus by the rat is enhanced by feeding high levels of vitamin D and by employing a favorable Ca : P ratio. In our studies in which we have shown utilization of soybean oil meal phosphorus (58%

[3] Stock ration consisted of wheat, corn, linseed oil meal, soybean oil meal, tankage, ground alfalfa, butter, liver powder, $CaCO_3$ and NaCl.

in the form of phytin or phytic acid), 70 U.S.P. units of vitamin D were fed weekly and favorable Ca : P ratios were maintained.

Extension of these studies to other species has demonstrated phytase in the mucosa of the small intestine of the chicken, pig and cow. Some activity was also observed in the intestinal contents of the pig and cow. Under practical conditions where sufficient vitamin D is obtained from solar irradiation it seems likely that these animals too would utilize phytin or phytic acid phosphorus since phytase is present in their intestinal mucosas. This would explain the results obtained by Hart et al. ('09) and Singsen and Mitchell ('44). According to Kreiger ('38) it seems quite possible that vitamin D is directly related to the activity of phytase.

No phytase activity could be measured in the rations used in these studies. The importance of plant phytases in the utilization of phytin and phytic acid phosphorus has been discussed by McCance and Widdowsen ('44), Mellanby ('44), Pedersen ('41) and Singsen and Mitchell ('44). Plant phytases that have been reported show optimum activity under slightly acid conditions. These may function in hydrolyzing phytin and phytic acid during certain cooking procedures or in the acid parts of the digestive tract. However, the importance of the plant phytases is probably over-emphasized.

The possible phytase activity of the intestinal flora should not be overlooked since the intestinal contents of the rat, pig and cow exhibited some activity.

SUMMARY AND CONCLUSIONS

It has been shown that soybean oil meal phosphorus, which was 58% phytin or phytic acid phosphorus, was utilized by the rat without a ration-borne heat labile phytase. Heating the rations at 98°C. for 48 hours did not depress the utilization of the phosphorus.

A method for the determination of phytase activity was developed which revealed the presence of a phytase in the small intestinal mucosa and in the intestinal contents of the rat. The optimum pH of this enzyme was found to be pH 7.8. Although the addition of magnesium ions to a crude enzyme preparation caused an increase in total phosphorus liberated, it did not stimulate the action of phytase. Phytase activity was observed in the small intestines of rats of all ages and in the small intestines of rats receiving several different rations.

Solvent or expeller soybean meals failed to show any phytase activity at pH 5.2 or at pH 7.8.

Phytase activity was observed in the small intestinal mucosa of the chicken, pig and cow.

From these studies it would appear that the phytin or phytic acid phosphorus of soybean oil meal was made available by the action of intestinal phytases.

LITERATURE CITED

ADLER, L. 1916 Gewinnung von Phytase aus Malz. Biochem. Z., vol. 75, p. 319.

FISKE, C. H., AND Y. SUBBAROW 1925 The colorimetric determination of phosphorus. J. Biol. Chem., vol. 66, p. 375.

HART, E. B., E. V. MCCOLLUM AND J. G. FULLER 1909 The role of inorganic phosphorus in nutrition of animals. Wisconsin Research Bull. 1.

HORIUCHI, K. 1931 Über die Phytase und die Spezifität der Phosphoesterase. J. Biochem. Tokyo, vol. 14, p. 163.

KAY, H. D. 1932 Phosphatase in growth and disease of bone. Physiol. Rev., vol. 12, p. 384.

KREIGER, C. H. 1938 Phytase and other phosphatases of the gastrointestinal tract. Thesis submitted for Master of Science degree University of Wisconsin.

KREIGER, C. H., AND H. STEENBOCK 1940 Cereals and rickets. XII. The effect of calcium and vitamin D on the availability of phosphorus. J. Nutrition, vol. 20, p. 125.

LOWE, J. T., AND H. STEENBOCK 1936 CLXI. Cereals and rickets. VIII. The hydrolysis of phytin in the intestines. Biochem. J., vol. 30, p. 1991.

MCCANCE, R. A., AND E. M. WIDDOWSEN 1944 Activity of the phytase in different cereals and its resistance to dry heat. Nature, vol. 153, p. 650.

MCCOLLUM, E. V., AND E. B. HART 1908 On the occurrence of a phytin-splitting enzyme in animal tissues. J. Biol. Chem., vol. 4, p. 497.

MELLANBY, E. 1944 Phytic acid and phytase in cereals. Nature, vol. 154, p. 394.

PATWARDHAN, N. V. 1937 LXXVIII. The occurrence of a phytin-splitting enzyme in the intestines of albino rats. Biochem. J., vol. 31, p. 560.

PEDERSEN, J. G. A. 1941 Experimental rickets in swine. The significance of the phytin and phytase content of the feed. Chem. Zentr., vol. 2, p. 1037.

PLIMMER, R. H. A. 1913 IV. The metabolism of organic phosphorus compounds. Their hydrolysis by the action of enzymes. Biochem. J., vol. 7, p. 43.

POTTER, V. R., AND C. A. ELVEHJEM 1936 A modified method for the study of tissue oxidations. J. Biol. Chem., vol. 116, p. 495.

RAPOPORT, S., E. LEVA AND G. M. GUEST 1941 Phytase in plasma and erythrocytes of various species of vertebrates. J. Biol. Chem., vol. 139, p. 621.

SINGSEN, E. P., AND H. H. MITCHELL 1944 Soybean meal chick rations need no inorganic phosphorus supplements. Poultry Sci., vol. 23, p. 152.

SPITZER, R. R., AND P. H. PHILLIPS 1945 The availability of soybean oil meal phosphorus to the rat. J. Nutrition, vol. 30, p. 117.

SUZUKI, U., K. YOSHINUIRA AND M. TAKAISKI 1906 Ueber ein Enzym phytase, "das, anhydro-oxy-methylendiphosphorsäure" spalet. Tokyo Imp. Univ. Coll. Agric. Bull., vol. 7, p. 503.

PHYSIOLOGICAL AVAILABILITY OF THE VITAMINS

III. THE EFFECT OF DIETARY ASCORBIC ACID OXIDASE [1]

MELVIN HOCHBERG, DANIEL MELNICK AND BERNARD L. OSER
Food Research Laboratories, Inc., Long Island City, New York

(Received for publication April 11, 1945)

Wokes and Organ ('43) have reviewed the literature on the presence of ascorbic acid oxidase in plant tissues and the rapidity with which it destroys the ascorbic acid present. The tests conducted by Hochberg, Melnick and Oser ('43) on a homogenized composite of a daily ration of foods commonly consumed by man indicate that the activity of the ascorbic acid oxidase derived from vegetables is not materially inhibited by the presence of other foods free from this enzyme. Complete conversion of reduced to dehydroascorbic acid occurred during the period required for the homogenization of the diet, even under conditions which would be expected to inhibit oxidation of the vitamin. In the presence of molecular oxygen the oxidation of ascorbic acid may proceed beyond the dehydro stage to diketogulonic acid (Levcowich and Batchelder, '42; Penney and Zilva, '43) to oxalic acid (Dodds and Gallimore, '32; Rosenfeld, '43) and possibly to other nutritionally inactive compounds.

Young and McHenry ('42) have drawn comparisons between the calculated and determined amounts of ascorbic acid in a series of meals. The analytical values were only 24 to 75% of the theoretical median as calculated from three sets of reference tables dealing with food composition. They point out that determinations of vitamin content of fresh foods may provide little information as to the amounts in the foods as consumed, since there may be variations in duration and conditions of storage, cooking procedures, and other factors. It seemed possible, therefore, that ascorbic acid oxidase, being responsible in large measure for the lower-than-expected ascorbic acid content of foods about to be eaten, may operate in vivo, thus reducing the physiological effectiveness of the ingested vitamin.

[1] Some of the results in this paper were presented in summary before the Division of Biological Chemistry at the 108th meeting of the American Chemical Society, New York, N. Y. The expenses of these studies were defrayed by a grant from Lever Brothers Company, Cambridge, Mass.

In the present study the influence of ascorbic acid oxidase upon the availability of dietary and extra ascorbic acid was investigated. In vitro experiments were also conducted. The photometric method of Hochberg, Melnick and Oser ('43) was employed for the determination of both dietary and urinary ascorbic acid, measurements being made of both reduced and dehydroascorbic acid. The test subjects were the same as those previously employed (Melnick, Hochberg and Oser, '45). The basal diet, however, was purposely modified to include a greater

TABLE 1

In vitro tests of the activity of dietary ascorbic acid oxidase.

EXPERIMENT		ASCORBIC ACID FOUND IN DIET		
No.	Conditions [1]	Reduced	Dehydro	Total
		mg.	*mg.*	*mg.*
I	Blanched,[2] homogenized basal diet	101	13	114
II	I incubated 6 hrs. at 37°C.	65	27	92
III	I + 200 mg. of ascorbic acid incubated 6 hrs. at 37°C.	240	39	279
IV	Unblanched, homogenized basal diet	0	112	112
V	IV incubated 6 hrs. at 37°C.	1	44	45
VI	IV + 200 mg. of ascorbic acid incubated 6 hrs. at 37°C.	128	78	206

[1] The homogenized mixtures were incubated at their natural pH of 5.5 in open beakers and were stirred several times every half hour.

[2] The solid items in the ration were subdivided into particles, approximately 1 cubic inch, and immediately dropped into the boiling milk (previously deaerated), and the boiling continued for a 20-minute period under nitrogen. The mixture was then cooled in an ice bath and homogenized, both under nitrogen. The pH of the suspension was 5.5 throughout.

amount of raw vegetables since they contribute ascorbic acid oxidase.[2] The reason for this change was to allow a more measurable destruction of the vitamin to occur in vivo if such should be the case. The modifications in the diet were planned to permit an increase in ascorbic acid oxidase content without changing the values for the proximate constituents or vitamin content.

EXPERIMENTAL PART

In vitro tests. In table 1 are presented the results of in vitro experiments on the activity of dietary ascorbic acid oxidase. Two series of tests were conducted, both on the homogenized basal diet. In one series,

[2] The ration included bread, butter, steak, milk, eggs, sugar, orange, banana, apple, lettuce, tomato, cucumber, and a salad of raw cabbage, green beans, pepper, and carrot.

the diet [3] was first blanched in order to inactivate all enzymes present. In the other, the unblanched homogenized ration was employed. The dietary mixtures were then incubated in open beakers for a period of 6 hours at 37°C. at their natural pH of 5.5, with gentle stirring every ½ hour.

The results summarized in table 1 indicate that during the incubation of a dietary mixture after heat-inactivation of all enzymes, small but appreciable loss of ascorbic acid occurred. That this was due to oxidation may be inferred from the fact that the dehydroascorbic acid value had increased. These losses in ascorbic acid appear to be absolute since added ascorbic acid (experiment III) was almost quantitatively recovered in the reduced state from the dietary mixture.

Simple homogenization of the unheated dietary mixture resulted in marked oxidation of the ascorbic acid to dehydroascorbic acid, but with only slight oxidation beyond that stage. However, during incubation of the dietary mixture at 37°C., fully 60% of the dehydroascorbic acid was converted to non-biologically active forms. When extra ascorbic acid was added to the dietary mixture, much of the added vitamin was oxidized to and some even beyond the dehydroascorbic acid stage.

The results of the in vitro tests support the hypothesis advanced by Green ('41) that ascorbic acid oxidase catalyzes the oxidation of ascorbic acid in an absolute manner, i.e., the rate of oxidation being independent of the concentration of the vitamin. Since factors are known to be present in the gastrointestinal tract which can destroy enzymes or render them inactive, it was necessary to determine by direct studies on man what effect the ascorbic acid oxidase might have on the availability of the vitamin when cell rupture occurs in the gastrointestinal tract.

In vivo study. In table 2 are shown the results of the study on human subjects. The control data were obtained with the basal ration previously heated to inactivate all enzymes present.

During the first 3 days no extra ascorbic acid was taken; on the fourth day a test dose of 200 mg. of the vitamin was administered; and on the fifth day the basal ration alone was again ingested. The responses of the subjects were quite uniform in respect to reproducibility in their

[3] The solid constituents of the ration were subdivided into small particles (about 1 cubic inch) and dropped immediately into boiling milk previously deaerated by a stream of nitrogen. The boiling was continued for 20 minutes. The mixture was then cooled in an ice bath and homogenized in a Waring blendor under nitrogen. In the case of the unblanched diet the homogenization was carried out under air.

basal ascorbic acid excretion, response to the test doses, and small carry-over (second 24-hour) excretions. Two weeks later the experiment was repeated, but this time the diet was consumed with the vegetables in their natural raw state. No initial or progressive decreases in the basal urinary excretion values were noted when the subjects received the unheated diet. After the postprandial test dose of 200 mg. of ascorbic acid, an increase in the urinary excretion of the vitamin resulted, followed by a small carry-over during the subsequent 24-hour period. The extra excretion of ascorbic acid following dosage was comparable to that observed during the control period when no active ascorbic acid oxidase remained in the foods taken.

TABLE 2

Urinary excretion of dietary and extra ascorbic acid by subjects receiving diets containing active and heat-inactivated[1] ascorbic acid oxidase.
(All values in milligrams per 24 hours.)

SUBJECT	DIET WITH ENZYMES HEAT-INACTIVATED				DIET CONTAINING ACTIVE ENZYMES				
	Basal excretions		After 200 mg. test dose of ascorbic acid		Basal excretions			After 200 mg. test dose of ascorbic acid	
	1st 24 hrs.	3rd 24 hrs.	1st 24 hrs.	2nd 24 hrs.	1st 24 hrs.	2nd 24 hrs.	3rd 24 hrs.	1st 24 hrs.	2nd 24 hrs.
J.C.	56	44	141	86	36	45	57	141	69
E.M.	52	60	195	88	74	61	59	172	94
D.M.	63	55	135	75	40	44	43	163	84
M.H.	51	53	165	90	67	59	57	199	97
H.H.	12	14	66	39	22	23	27	103	42
Average	47	45	140	76	48	46	48	156	77

[1] The enzymes in the diet were inactivated by heat essentially as in the in vitro studies (see footnote to table 1) except that the blanched mixture was not homogenized.

The values shown in table 2 are for total ascorbic acid. However, in the course of the urine analyses, reduced ascorbic acid (and by difference, dehydroascorbic acid) was also determined. These data are not included in table 2 since they indicate no significant difference in the urinary excretion of either form of the vitamin during the control and test periods. In all cases approximately 91% of the total vitamin in the urine was reduced ascorbic acid, the remainder being in the dehydro state. This is in fairly good agreement with the 82% figure reported by Berryman and associates ('44) for the fraction found in the reduced form in urine after dosage with the reduced ascorbic acid.

In table 3 the data of table 2 are restated in terms of per cent of test dose excreted when taken with the diets containing active and heat-

inactivated enzymes, respectively. In considering these data it should be pointed out that somewhat greater individual variations were observed from control to test period than in the case of other ascorbic acid assays conducted with the same subjects. However, group averages are reproducible (Melnick, Hochberg and Oser, '45). The average 48-hour extra excretion of ascorbic acid when the added vitamin was consumed along with the diet in which all enzymes had been heat-inactivated was found to be 63% of the dose. A value of 71% was obtained when the vitamin was taken with the natural diet, i.e., in the presence of active ascorbic acid oxidase. The difference is not statistically significant ($t = 0.84$). It is apparent from these data that no demonstrable

TABLE 3

Stability in vivo of dietary and extra ascorbic acid in the presence of natural ascorbic acid oxidase.

SUBJECT	DIET WITH ENZYMES HEAT-INACTIVATED			DIET CONTAINING ACTIVE ENZYMES		
	Average basal excretion	Fraction of 200 mg. test dose excreted		Average basal excretion	Fraction of 200 mg. test dose excreted	
		1st 24 hrs.	2nd 24 hrs.		1st 24 hrs.	2nd 24 hrs.
	mg./24 hrs.	%	%	*mg./24 hrs.*	%	%
J.C.	50	46	18	46	48	12
E.M.	56	70	16	65	54	15
D.M.	59	38	8	42	61	21
M.H.	52	57	19	61	69	18
H.H.	13	27	13	24	40	18
Average	46	48	15	48	54	17
Average total excretion of extra ascorbic acid		62.4%			71.2%	
Availability of ascorbic acid in presence of oxidase		$\frac{71.2}{62.4} \times 100 = 114 \pm 16\%$				

destruction of ascorbic acid occurred by virtue of the concomitant ingestion of active ascorbic acid oxidase. In fact two of the subjects (D.M. and H.H.) present the apparent anomaly of excreting somewhat more ascorbic acid when the active enzymes were included in the diet. However, the excretion values for these particular individuals during the control period (heat-inactivated diet) were somewhat lower than usual.

The in vitro tests (table 1) demonstrated a proportionately greater destruction to have occurred in the naturally supplied ascorbic acid because the enzyme appeared to operate in an absolute rather than a relative manner. However, the reproducibility of the basal ascorbic

acid values in this study from one period to the other also demonstrates inactivity of ascorbic acid oxidase in vivo.

It is conceivable that in the gastrointestinal tract some oxidation of ascorbic acid to dehydroascorbic acid may have occurred since this change would not be detected by the assay technic. No differences occur in the form and amount of ascorbic acid excreted in the urine following dosage with reduced or dehydroascorbic acid (Johnson and Zilva, '34). The inability to determine by the bioassay technic this "incipient" oxidation of reduced ascorbic acid is unimportant nutritionally inasmuch as the organism can utilize dehydroascorbic acid equally well.

SUMMARY

In vitro tests have emphasized the rapidity with which the ascorbic acid oxidase in vegetables can oxidize ascorbic acid to and beyond the dehydroascorbic stage. Simple incubation of the dietary mixture at body temperature for a period of 6 hours resulted in a loss of approximately 60% of the biologically active vitamin. The amount of enzyme present in the homogenized diet was sufficient to catalyze oxidation of added ascorbic acid to and beyond the dehydroascorbic acid stage.

The results of the human availability study indicate that no greater destruction of either the naturally occurring ascorbic acid or of the extra ascorbic acid ingested takes place in vivo prior to absorption. Apparently ascorbic acid oxidase is destroyed or its activity is inhibited in the gastrointestinal tract. Oxidation of the vitamin to dehydroascorbic acid may have occurred but in view of the biological activity of this partially oxidized form of the vitamin, this is of no importance nutritionally.

LITERATURE CITED

BERRYMAN, G. H., C. E. FRENCH, H. A. HARPER AND H. POLLACK 1944 Response to the intravenous injection of ascorbic acid as indicated by the urinary excretion of the total and reduced forms. J. Nutrition, vol. 27, p. 309.

DODDS, E. C., AND E. J. GALLIMORE 1932 The determination of small quantities of oxalic acid. Biochem. J., vol. 26, p. 1242.

GREEN, D. E. 1941 Mechanism of Biological Oxidations. Cambridge University Press, London, England.

HOCHBERG, M., D. MELNICK AND B. L. OSER 1943 Photometric determination of reduced and total ascorbic acid. Ind. Eng. Chem., Anal. Ed., vol. 15, p. 182.

JOHNSON, S. W., AND S. S. ZILVA 1934 The urinary excretion of ascorbic and dehydroascorbic acids in man. Biochem. J., vol. 28, p. 1393.

LEVCOWICH, T., AND E. L. BATCHELDER 1942 Ascorbic acid excretion at known levels of intake as related to capillary resistance, dietary estimates, and human requirements. J. Nutrition, vol. 23, p. 399.

MELNICK, D., M. HOCHBERG AND B. L. OSER 1945 Physiological availability of the vitamins. I. The human bioassay technic. J. Nutrition, vol. 30, p. 67.

PENNEY, J. R., AND S. S. ZILVA 1943 The determination of 2, 3-diketo-l-gulonic acid. Biochem. J., vol. 37, p. 39.

ROSENFELD, B. 1943 The irreversible transformation of dehydroascorbic acid. J. Biol. Chem., vol. 150, p. 281.

WOKES, F., AND J. C. ORGAN 1943 Oxidizing enzymes and vitamin C in tomatoes. Biochem. J., vol. 37, p. 259.

YOUNG, C. M., AND E. W. MCHENRY 1942 Errors in the calculation of the nutritive value of food intake. II. Comparison of calculated and determined amounts of ascorbic acid. Can. Pub. Health J., vol. 33, p. 224.

PHYSIOLOGICAL AVAILABILITY OF THE VITAMINS

IV. THE INEFFICIENCY OF LIVE YEAST AS A SOURCE OF THIAMINE [1]

MELVIN HOCHBERG, DANIEL MELNICK AND BERNARD L. OSER
Food Research Laboratories, Inc., Long Island City, New York

(Received for publication April 11, 1945)

Live compressed yeast has been widely advertised as a good source of the vitamin B complex. However, studies by Walker and Nelson ('33) showed that vitamin B in fresh yeast is not readily available for absorption in the gastrointestinal tract of the rat due presumably to the resistance of the live yeast cell to digestion (Montgomery and associates, '31).

In chemical methods of assay, the cells are ruptured by boiling the suspension of the yeast in dilute sulfuric acid, making possible measurement of the total thiamine content. We have confirmed this by rat assays (curative) of neutralized acid extracts of yeast.

Parsons and associates ('42 b) at the University of Wisconsin presented evidence of the non-availability to man of the water-soluble vitamins in fresh yeast. When the yeast was suspended in boiling water, and then fed, evidence of thiamine absorption was obtained. In only one type of live compressed yeast was the thiamine present in utilizable form (Parsons and collaborators, '45). This sample contained five times as much thiamine, but otherwise was identical with one of the yeast cakes previously shown to contain thiamine in a non-available form. The authors concluded that the extent to which thiamine was available in live yeast was determined not by the strain of yeast, but by the high concentration of thiamine in the yeast cells.

In the present study, the availability to man of thiamine in the live yeast was investigated by means of the human availability technic described by Melnick, Hochberg and Oser ('45). Thiamine intake was determined by the thiochrome procedure (Hennessy, '41), and urinary excretion by the colorimetric method (Hochberg and Melnick, '44).

[1] Some of the results in this paper were presented in summary before the Division of Biological Chemistry at the 108th meeting of the American Chemical Society, New York, N. Y. The expenses of these studies were defrayed by a grant from Lever Brothers Company, Cambridge, Mass.

Thiamine was the only vitamin included in this study, since according to the work of Parsons and associates ('42) its excretion is an index of the degree of availability of all the water-soluble vitamins present. Parallel in vitro studies were also conducted.

In vitro tests

It has long been known that, during growth and multiplication, yeast cells are able to remove thiamine from solution. Thus, brewers' yeast is rich in thiamine while the beer contains only a small fraction of the vitamin originally present in the wort. Thiamine is present in yeast primarily in the phosphorylated form. During the drying of yeast cells a phosphatase is liberated which converts some of the combined thiamine to the free form (Melnick and Field, '39). Therefore aid-dried yeast samples may contain variable proportions of thiamine in the phosphorylated state, depending on the conditions of preparation. In the present report the differences in the thiamine values before and after treatment of the test extracts with phosphatase are attributed to the presence of phosphorylated thiamine.

TABLE 1

Free and phosphorylated thiamine in commercial samples of moist compressed yeast. (All values in micrograms per gram.)

SAMPLE		THIAMINE FOUND			
No	Description	Non-phosphorylated	Phosphorylated	Total	Per cent Phosphorylated
1	Regular yeast	0.3	3.9	4.2	93
2	Regular yeast	0.5	4.6	5.1	90
3	High vitamin yeast	50	164	214	77
4	High vitamin yeast	47	152	199	76
5	High vitamin yeast	64	13	77	17

In table 1 are presented the results obtained in the analyses of five samples of compressed yeast cakes. Two of these were bakers' yeast, available for commercial use but not enriched with added vitamins. Preparations 3 and 4 were "high vitamin" yeast preparations employed at the time in the manufacture of enriched bread. The fifth sample was a high vitamin yeast cake sold directly to the public as a source of the water-soluble vitamins. The data indicate that, in the non-enriched yeast samples, approximately 90% of the thiamine was present in the phosphorylated state. In the high vitamin yeast used for baking purposes, about three-fourths of the vitamin was found in the

combined form. In the sample intended for direct human consumption, only about one-sixth of the vitamin was phosphorylated.

In table 2 are presented the results of experiments to determine the fate of thiamine added to an aqueous suspension of live yeast cells. For these experiments, the non-enriched variety of yeast was employed. Six yeast cakes weighing 75 gm. were dispersed in 360 ml. of acetate buffer at pH 4.5 to make a uniform suspension. This was then stored at 37°C. for 6 hours with rotatory agitation every ½ hour. At the end of the 6-hour period the suspensions were centrifuged and assays conducted on the washed yeast sediment and on the pooled supernatant

TABLE 2

Absorption and phosphorylation of thiamine by yeast cells[1] in aqueous suspension.

SAMPLE	THIAMINE FOUND, PER ENTIRE SAMPLE			
	In solution	In yeast sediment		
	Non-phosphorylated	Non-phosphorylated	Phosphorylated	Total
75 gm. yeast[2]	12 μg. 4%	10 μg. 3%	290 μg. 93%	300 μg. 96%
75 gm. yeast + 1000 μg. thiamine	7 μg. 0.5%	36 μg. 3%	1152 μg. 97%	1168 μg. 100%

[1] Sample no. 1 of table 1.

[2] The weight of 6 commercial yeast cakes is 75 gm. Uniform suspensions in 360 ml. of acetate buffer at pH 4.5 were prepared and stored at 37°C. for 6 hours. These were stirred every ½ hour. The suspensions were centrifuged and assays conducted on the washed yeast sediment and on the pooled supernatant phase plus washings.

phase plus the washings. These tests were repeated on another sample of the suspended yeast cells to which had been added 1000 μg. of synthetic free thiamine.

Practically all the vitamin originally in solution was found to be removed by the yeast cells. Furthermore, almost all of the thiamine in the yeast sediment was in the phosphorylated state. This removal of thiamine from solution and its phosphorylation by the live yeast cell is confirmatory of the results obtained in these laboratories on the fate of thiamine added for enrichment of bread (Melnick and associates, '41). It was found that during the fermentation of the dough, yeast phosphorylated the synthetic free thiamine added for enrichment, so that the baked bread contained the same percentage of the total thiamine in the phosphorylated form[2] as when natural combined thiamine was used for the fortification.

[2] Confirmation that this was indeed phosphorylated thiamine was obtained by testing specifically for cocarboxylase by the Lohmann and Schuster ('37) mannometric procedure.

The present experiments on the phosphorylating ability of yeast are of particular value since they definitely indicate that the thiamine removed from solution is not merely adsorbed on the cell wall but actually passes into the cell proper. This would seem to explain in part the mechanism responsible for the low availability of thiamine to human subjects as reported by Parsons and associates ('42a, '42b). Because better availability was observed by the Wisconsin workers (Parsons and associates, '45) from live yeast containing high proportions of thiamine, the in vitro tests were extended to studies of the enriched yeast sample recommended for direct consumption. From the values given in table 1, it was apparent that this yeast differed from the others in that most of its thiamine was present in the free state.[3] Analyses were then conducted on the suspension of the live yeast cells before and after the period of incubation at 37°C. Furthermore, the vitamin in solution was partitioned into free and phosphorylated thiamine.

The results shown in table 3 demonstrate that in the supernatant solution practically all the vitamin was present in the free state. During the period of storage at 37°C., the proportion of thiamine retained by the yeast sediment increased from 28 to 52%. This was due to a rise in concentration of both the free and phosphorylated vitamin. When extra thiamine was added to the suspension of live yeast cells no increase was observed in the absolute quantity of thiamine in the yeast sediment, either as the free or phosphorylated vitamin.

The presence of the thiamine in this yeast sample primarily as the free vitamin, the inability of these live yeast cells to phosphorylate thiamine to any significant extent and the large overage in thiamine content (i.e., above that indicated on the label) suggested that at least the claimed quantity of thiamine in the product might be available to the human. It was suspected that this might represent an attempt on the part of the manufacturer to solve the problem of the unavailability of thiamine in the live yeast cell. These factors might explain the favorable results obtained by Parsons and associates ('45) when live yeast cells containing large quantities of thiamine were fed.

In vivo tests

Human availability studies were conducted on the yeast sample used in the experiment summarized in table 3. Six yeast cakes (each 12.5 gm.) were taken, suspended in milk, two after each meal. During the control

[3] The actual thiamine content of the sample as determined by chemical analysis was almost twice as great as claimed for the product, namely, 1.92 mg. instead of 1.0 mg. per two yeast cakes.

TABLE 3

In vitro absorption and phosphorylation of thiamine by enriched live yeast [1] in aqueous suspension.

EXPERIMENT	THIAMINE FOUND, PER ENTIRE SAMPLE					
	In solution			In yeast sediment		
	Non-phosphorylated	Phosphorylated	Total	Non-phosphorylated	Phosphorylated	Total
75 gm. yeast [2]	.....	.....	.....	4.80 mg.	0.96 mg.	5.76 mg.
				83%	17%	100%
75 gm. yeast suspended in acetate buffer at pH 4.5 at 25°C. for 2 min.	3.90 mg.	0.09 mg.	3.99 mg.	1.08 mg.	0.45 mg.	1.53 mg.
	71%	2%	72%	20%	8%	28%
75 gm. yeast suspended in acetate buffer at pH 4.5 and stored at 37°C. for 6 hrs.	2.55 mg.	0.36 mg.	2.91 mg.	2.34 mg.	0.84 mg.	3.18 mg.
	42%	6%	48%	38%	14%	52%
75 gm. yeast + 5.76 mg. thiamine suspended in acetate buffer at pH 4.5 and stored at 37°C. for 6 hrs.	7.59 mg.	0.30 mg.	7.89 mg.	2.46 mg.	0.78 mg.	3.24 mg.
	68%	3%	71%	22%	7%	29%

[1] Sample no. 5 of table 1. [2] Six yeast cakes.

TABLE 4

Rate and degree of urinary excretion of thiamine following ingestion of the vitamin in solution and in yeast.

(All values in milligrams per day.)

SUBJECT	THIAMINE IN SOLUTION			THIAMINE IN YEAST CAKES		
	Basal	After 5.76 mg. of thiamine in solution [1]		Basal	After 6 live yeast cakes [2]	
		1st 24 hrs.	2nd 24 hrs.		1st 24 hrs.	2nd 24 hrs.
J.C.	0.26	1.78	0.57	0.23	0.70	0.40
E.M.	0.20	1.67	0.64	0.28	0.37	0.29
D.M.	0.20	1.56	0.55	0.25	0.44	0.25
M.H.	0.20	1.68	0.59	0.24	0.56	0.37
H.H.	0.17	1.48	0.45	0.21	0.30	0.22
Average	0.21	1.63	0.56	0.24	0.47	0.31

[1] Taken as three 1.92 mg. doses of thiamine in milk, one dose at the end of each meal.

[2] Containing 5.76 mg. of thiamine. Two cakes, containing 1.92 mg., were suspended in milk and taken at the end of each meal.

period, the same quantity of pure thiamine was taken dissolved in milk, one-third of the dose being given after each meal. Each dose was 1.92 mg. of thiamine, the total being 5.76 mg. per subject per day. The results obtained in this study are summarized in table 4.

The basal urinary excretion values for the five test subjects were of the same magnitude during the control and test periods. However, the marked increases in the urinary excretion values following the taking of thiamine in solution were not observed when the test dose of thiamine was taken as the live yeast supplement. Inasmuch as one dose was taken after the evening meal, it was considered advisable to collect the urine samples for an additional 24-hour period in order to be sure that an erroneous conclusion regarding availability would not

TABLE 5

Availability of thiamine in yeast.

SUBJECT	PORTION OF TEST DOSE EXCRETED					
	Control period, thiamine in solution			Test period, thiamine in yeast		
	1st 24 hours	2nd 24 hours	Total 48 hours	1st 24 hours	2nd 24 hours	Total 48 hours
	%	%	%	%	%	%
J.C.	26.4	5.4	31.8	8.2	3.0	11.2
E.M.	25.5	7.6	33.1	1.6	0.2	1.8
D.M.	23.6	6.1	29.7	3.3	0	3.3
M.H.	25.7	6.8	32.5	5.6	2.3	7.9
H.H.	22.7	4.9	27.6	1.6	0.2	1.8
Average	24.8	6.2	30.9	4.1	1.1	5.2

Availability of thiamine in yeast $= \frac{5.2}{30.9} \times 100 = 17\%$.

be due simply to a slower rate of absorption of the thiamine derived from the intact yeast cells. Examination of the data indicates that the "carry-over" effect was not responsible for the lower thiamine excretion during the first 24 hours.

In table 5, the results in the preceding table are restated on a percentage excretion basis. The urinary values demonstrate that approximately 25% of the pure solution dose was excreted during the 24 hours following the taking of the first supplement, and 6% more during the second 24-hour period for a total excretion of 31%. After ingestion of the live yeast cakes, the quantities of extra thiamine found in the urine were exceedingly small; 4% of the dose during the first 24 hours and 1% during the second 24 hours. Thus, the physiologically available thiamine in live yeast is calculated to be only 17%, or 33% of that

claimed for this particular product, despite the fact that the greater portion of its thiamine was in the free state and that the material exhibited a markedly limited capacity to phosphorylate the vitamin.

The low availability cannot be attributed to a slower rate of absorption of the thiamine from live yeast with concomitant improvement in its utilization. If this were the case, one would expect the urinary response following administration of a pure solution of thiamine in three separate test doses over a period of a day to be less than that noted when the same quantity of the vitamin was taken as a single test dose. Such was not the case.[4] Furthermore, the ratio of the urinary excretion responses after taking the thiamine in the form of live yeast as compared to taking thiamine in solution during the first 24 hours, namely, 4 to 25, was exactly the same as the ratio of the carry-over values, 1 to 6%.

SUMMARY

Bakers' yeast is capable of removing large quantities of thiamine from solution. That this removal is not due to simple adsorption of the thiamine on the yeast cell wall but to actual passage of the thiamine into the cell proper, is evidenced by phosphorylation of the vitamin. Other preparations of live yeast on the market have limited ability to phosphorylate thiamine. In these samples the major portion of the thiamine is found in the free state.

Despite the facts that (a) most of the thiamine in the live yeast employed in the present human availability study was present in the free form, (b) the yeast was practically ineffective in phosphorylating the vitamin and (c) there was almost a 100% overage of thiamine in the sample — all of which should favor the presence of available thiamine at least to the extent of the content of thiamine claimed for the product — only 17% of the total thiamine present was physiologically available, or 33% of the claimed potency.

The low availability value for the thiamine could not be attributed to a slower rate of absorption of the vitamin from live yeast.

ADDENDUM

Correspondence with Dr. Helen T. Parsons, University of Wisconsin, Madison, confirmed the suspicion that the yeast sample used in our availability studies was made by the same manufacturer as her sample A_2. Calculations, similar to those used in the present studies indicate that 27% of the thiamine in her yeast sample A_2 is available (see table 3, Parsons and associates, '45). Considering that different test subjects were employed subsisting on basal rations which were not

[4] See previous report (Melnick, Hochberg and Oser, '45) presenting the excretion data when thiamine was taken postprandially as a single 5.0 mg. test dose.

the same, and that different batches of the yeast were used in the assays, the values obtained by the two laboratories are considered to be in very good agreement.

LITERATURE CITED

HENNESSY, D. J. 1941 Chemical methods for the determination of vitamin B_1. Ind. Eng. Chem., Anal Ed., vol. 13, p. 216.

HOCHBERG, M., AND D. MELNICK 1944 A simple modification of the colorimetric method for routine thiamine clearance tests. J. Biol. Chem., vol. 156, p. 53.

LOHMANN, K., AND P. SCHUSTER 1937 Untersuchungen über die Cocarboxylase. Biochem. Ztschr., vol. 294, p. 188.

MELNICK, D., AND H. FIELD, JR. 1939 Chemical determination of vitamin B_1. Hydrolysis of cocarboxylase. J. Biol. Chem., vol. 127, p. 531.

MELNICK, D., M. HOCHBERG AND B. L. OSER 1945 Physiological availability of the vitamins. I. The human bioassay technic. J. Nutrition, vol. 30, p. 67.

MELNICK, D., A. MABARDIE, A. BERNSTEIN AND B. L. OSER 1941 The fate of vitamin B_1 in production of baked goods. Northwestern Miller, vol. 208, p. 17.

MONTGOMERY, B. E., A. K. BOAR, L. ARNOLD AND O. BERGEIM 1931 Destruction of yeast in the normal human stomach. Proc. Soc. Exp. Biol. Med., vol. 28, p. 385.

PARSONS, H. T., AND J. COLLORD 1942 a Human utilization of thiamin and riboflavin in yeast. J. Am. Diet. Assn., vol. 18, p. 805.

PARSONS, H. T., J. COLLORD, J. GARDNER, F. M. STRONG AND W. H. PETERSON 1942 b Utilization by man of the vitamin B complex in fresh yeast. Federation Proc., vol. 1, p. 129.

PARSONS, H. T., A. WILLIAMSON AND M. L. JOHNSON 1945 The availability of vitamins from yeasts. I. The absorption of thiamine by human subjects from various types of bakers' yeast. J. Nutrition, vol. 29, p. 373.

WALKER, R., AND E. M. NELSON 1933 Fresh and dried yeast as sources of vitamin B. Am. J. Physiol., vol. 103, p. 25.

ESTIMATION OF THE RELATIVE NUTRITIVE VALUE OF VEGETABLE PROTEINS BY TWO CHEMICAL METHODS [1]

ROBERT JOHN EVANS AND J. L. ST. JOHN
Division of Chemistry, Washington Agricultural Experiment Station, Pullman, Washington

(Received for publication May 25, 1945)

Recent work of Draper and Evans ('44) has shown that different soybean oil meals may differ in protein quality as measured by the gross value method.

Almquist, Stokstad, and Halbrook ('35) developed a chemical method for estimating the quality of animal protein concentrates. Almquist ('41) stated that he did not recommend this method for vegetable protein concentrates since he had not investigated vegetable products. Olsen and Palmer ('40) compared the chemical protein quality index of Almquist with a biological value determined by the nitrogen retention method with rats. Their results with a number of animal and plant foods seem to indicate a relationship between the two methods for natural foods.

The simple vegetable proteins have been classified by Osborne ('24) according to their solubilities into the albumins (soluble in water), the globulins (insoluble in water but soluble in saline solutions), the glutelins (insoluble in water, saline solutions, or alcohol, but soluble in very dilute acids and alkalies), the prolamines (soluble in relatively concentrated alcohol), the albuminoids, the histones, and the protamines. To the knowledge of the authors, no attempt has been made to relate the relative amounts of these different proteins in a concentrate to the protein nutritive value of the concentrate.

It appeared desirable to study the relation of the nutritive values of some vegetable protein concentrates to the chemical protein quality index, and to the per cent of total nitrogen present in each of the different protein fractions defined above. It also appeared desirable to determine the influence of heat treatment upon the solubility of the soybean proteins. A determination of any chemical differences existing

[1] Published as Scientific Paper no. 653, College of Agriculture and Agricultural Experiment Station, State College of Washington, Pullman.

between the vegetable protein concentrates having high protein nutritive values and those having low protein nutritive values would be of importance from both theoretical and practical standpoints. A rapid method of estimating the relative quality of vegetable protein concentrates would also be of value.

EXPERIMENTAL

The gross protein values of ten soybean oil meals, two cottonseed meals, and two pea meals were determined with chicks by Draper and Evans ('44). The soybean oil meals were commercial products, obtained from three different sources and were prepared by expeller process, solvent process, or solvent process-uncooked. The method of preparation of two of the soybean oil meals was unknown, but from physical characteristics of the meals, they appear to have been prepared by the solvent process. The cottonseed meals were commercial products of unknown history. The pea meals were prepared by grinding cull Alaska, and First and Best peas.

Relation of chemical protein quality index to nutritive value

The chemical protein quality index was determined on these same vegetable protein concentrates by the procedure of Almquist, Stokstad, and Halbrook ('35). This method consists of a chemical determination of four protein fractions. The chemical protein quality index is calculated from these results by the following formula:

Protein quality index = A − (B + 0.6 C) + 0.4 D: Where A = per cent protein precipitated by copper salts (inclusive of B and C); B = per cent protein not digested with pepsin; C = per cent protein soluble in hot water; and D = per cent protein precipitated by phosphotungstic acid.

The values for the chemical protein quality index of the soybean oil meals ranged from 84.8 to 89.1 (table 1). Only one of these values was below 88.0. The values for the gain in weight per gram of supplementary protein consumed ranged from 3.5 to 6.6 gm. It is apparent that the growth method gave much larger differences than the chemical method. A coefficient of correlation of + 0.367 between the chemical index of protein quality and the gain per gram of supplementary protein consumed was obtained for all of the soybean oil meals. A coefficient of 0.632 was required for significance.

The two pea meals gave as good gains in weight per gram of supplementary protein as the expeller process soybean oil meals. The chemical protein quality indexes were definitely lower for the pea meals than

for the expeller process soybean oil meals. This was the result of the high hot-water-soluble protein fraction in the pea meals.

The poorest gains in weight per unit of supplementary protein were obtained with the cottonseed meals. The protein quality indexes were also the lowest. The low protein quality index of the cotttonseed meal was the result of a large indigestible protein fraction.

A coefficient of correlation between gain per gram of supplementary protein and the chemical protein quality index of + 0.815 was obtained for all of the vegetable protein concentrates of table 1. A value of 0.661 was required to be highly significant. Leaving the three expeller process soybean oil meals out of the calculations, a coefficient of correlation of + 0.946 was obtained while 0.735 was required to be highly significant.

No differences in chemical protein quality index between expeller and solvent process soybean oil meals were observed. The solvent process meals, however, gave a greater gain in weight per gram of supplementary protein than did the expeller process meals (table 1).

Relation of protein solubility to nutritive value

The various proteins, from samples of the same vegetable protein concentrates, were peptized and the per cent of total nitrogen in each fraction determined by the method of Lund and Sandstrom ('43). The proteins of each concentrate are divided by this method into the protein soluble in water (albumins and part of globulin), the protein soluble in 5% potassium chloride solution (remainder of globulins), the protein soluble in 70% ethyl alcohol solution (prolamines), the protein soluble in 0.2% potassium hydroxide solution (glutelins), and the residual protein fractions. They will be referred to as albumin, globulin, prolamine, glutelin, and residual proteins respectively in the remainder of this paper. Part of the globulins are included in the albumin fraction because of the presence of salts in the soybean oil meal.

The per cent of total protein present as albumins, globulins, prolamines, and glutelins for each sample is presented in table 2. The per cent of residual protein and the gain per unit of supplementary protein are also included in table 2. Over half of the protein of the uncooked concentrates was in the albumin fraction. The peas contained considerable protein in the globulin fraction.

There was little difference between any of the cooked meals in their contents of albumins, globulins, or prolamines. Less than 10% of the total nitrogen was in the albumin fraction; less than 5% was in the globulin fraction; and less than 4% was in the prolamine fraction.

TABLE 1

Comparison of the chemical protein quality index and the gain per gram of supplementary protein for some vegetable protein concentrates.

SAMPLE NO.	PROTEIN CONCENTRATE	PROCESS	GAIN PER UNIT PROTEIN [1]	CRUDE PROTEIN	PER CENT OF TOTAL PROTEIN				PROTEIN QUALITY INDEX
					Copper ppt. (A)	Phosph. tung. (D)	Undigest (B)	Hot water soluble (C)	
			gm.	*%*					*A − (B+.6C) − .4D*
565	Soybean oil meal	Unknown	6.6	46.3	98.7	0.6	6.0	5.4	88.1
758	Soybean oil meal	Unknown	6.4	46.8	97.6	0.9	5.1	5.1	89.7
635	Soybean oil meal	Solvent	6.4	48.6	97.4	0.8	6.6	5.4	88.6
763	Soybean oil meal	Solvent	6.2	46.8	96.9	0.9	6.4	4.9	88.4
667	Soybean oil meal	Solvent	5.7	46.8	97.2	0.6	5.8	4.3	89.1
706	Soybean oil meal	Solvent	5.7	47.1	96.6	0.8	5.5	4.9	88.5
759	Soybean oil meal	Solvent — uncooked	4.9	50.0	95.7	0.8	6.6	7.8	84.8
764	Soybean oil meal	Expeller	4.9	46.1	96.5	0.9	5.0	5.9	88.5
704	Soybean oil meal	Expeller	4.6	45.3	96.0	0.7	5.3	4.4	88.3
765	Soybean oil meal	Expeller	3.5	46.6	96.4	0.9	4.7	6.0	88.4
761	Cottonseed meal	Unknown	2.8	37.1	94.2	0.8	17.2	6.5	73.3
702	Cottonseed meal	Unknown	1.2	45.0	93.3	0.7	18.9	6.7	70.7
755	Alaska peas	Uncooked	4.7	23.4	90.6	1.7	3.8	12.8	79.9
754	First and best peas	Uncooked	4.5	27.7	92.2	1.1	3.6	18.4	77.6

[1] From Draper and Evans ('44).

TABLE 2

Protein distribution in some vegetable protein concentrates.

SAMPLE NO.	PROTEIN CONCENTRATE	PROCESS	GAIN PER GRAM OF PROTEIN [2]	ALBUMINS [1]	GLOBULINS [1]	PROLAMINES [1]	GLUTELINS [1]	RESIDUAL PROTEIN [1]
			gm.	%	%	%	%	%
565	Soybean oil meal	Unknown	6.6	8.9	3.6	3.4	45.3	38.7
758	Soybean oil meal	Unknown	6.4	9.9	4.7	3.7	50.0	30.9
635	Soybean oil meal	Solvent	6.4	7.6	2.9	2.0	47.5	40.2
763	Soybean oil meal	Solvent	6.2	6.7	1.7	1.9	43.4	46.1
667	Soybean oil meal	Solvent	5.7	8.7	2.7	2.3	49.1	38.1
706	Soybean oil meal	Solvent	5.7	7.9	4.2	4.2	45.2	37.7
759	Soybean oil meal	Solvent — uncooked	4.9	75.6	5.7	3.5	6.0	9.2
764	Soybean oil meal	Expeller	4.9	9.1	1.7	2.1	34.3	52.8
704	Soybean oil meal	Expeller	4.6	6.5	2.0	2.4	26.2	62.5
765	Soybean oil meal	Expeller	3.5	9.1	1.3	2.3	31.6	54.0
761	Cottonseed meal	Unknown	2.8	8.0	4.0	1.9	8.3	77.2
702	Cottonseed meal	Unknown	1.2	8.4	19.3	2.4	9.1	60.2
755	Alaska peas	Uncooked	4.7	56.1	17.8	3.9	4.3	17.4
754	First and best peas	Uncooked	4.5	53.7	20.6	3.7	5.5	15.4

[1] Per cent of total protein.

[2] From Draper and Evans ('44).

Eight and three-tenths per cent of the total nitrogen of one sample of cottonseed meal was in the glutelin fraction. Seventy-seven and two-tenths per cent of the nitrogen of this same meal occurred in the residual protein fraction. Fifty per cent of the nitrogen of one soybean oil meal sample was in the glutelin fraction, and only 30.9% occurred in the residual protein fraction. There was a highly significant correlation for the cooked meals of + 0.928 between the grams gain in weight of chicks per gram of supplementary protein consumed and the per cent of the total protein in the glutelin fraction. There was a coefficient of correlation of — 0.788 between the gain per gram of supplementary protein and the per cent residual protein in the cooked meals. A coefficient of correlation of 0.735 was required to be highly significant.

The solvent process meals contained a higher percentage of dilute alkali soluble protein and a lower percentage of residual protein than the expeller process meals, (table 2).

The influence of heat treatment on the protein distribution in solvent process soybean flakes

Portions of soybean oil meal sample no. 759, which was an uncooked solvent process meal, were given different heat treatments. Portions

TABLE 3

The influence of heat treatment on the protein distribution in solvent process soybean flakes.

HEAT TREATMENT	TIME HEATED	ALBUMINS [1]	GLOBULINS [1]	PROLAMINES [1]	GLUTELINS [1]	RESIDUAL PROTEIN [1]
	min.	%	%	%	%	%
None	0	75.6	5.7	3.5	6.0	9.2
Autoclaved at 121°C.	5	49.6	6.3	2.7	19.8	21.6
Autoclaved at 121°C.	15	8.8	6.4	3.4	45.8	35.9
Autoclaved at 121°C.	30	6.4	1.6	3.2	38.6	49.6
Autoclaved at 121°C.	60	7.5	1.2	2.6	22.6	65.4
Autoclaved at 121°C.	120	8.6	1.0	2.7	10.0	77.3
Heated dry at 121°C.	60	64.4	6.8	2.3	10.1	16.5
Cooked in boiling water	60	15.2	3.6	3.6	38.2	38.6
Autoclaved at 110°C.	60	6.1	2.2	3.0	44.2	43.6

[1] Per cent of total protein.

were autoclaved at 121°C. for 5, 15, 30, 60, and 120 minutes. One portion was heated dry at 121°C., another was cooked wet by heating in a beaker in a boiling water bath, and another was autoclaved at 110°C. for 60 minutes. The proteins were divided into five fractions as described under the preceding section.

The results are presented in table 3. Heating decreased the percentage of albumins and globulins present in the soybean oil meal. The

glutelins were first increased and then decreased by heat treatment. The residual or insoluble protein was increased by heat treatment. It is apparent from the changes in solubility caused by heating, that the glutelin and residual protein fractions were composed largely of denatured proteins. A slight heat denaturation produced a protein insoluble in water or 5% potassium chloride, but soluble in 0.2% potassium hydroxide. Further heat denaturation produced a protein that was insoluble in 0.2% potassium hydroxide.

Dry heat was less effective than wet heat in causing a heat denaturation of the soybean proteins. The amount of heat denaturation was increased both by increasing the temperature of heating and increasing the time of heating.

DISCUSSION

The chemical protein quality index was found satisfactory as a chemical method of evaluating the protein nutritive values of animal protein concentrates (Almquist, Stokstad, and Halbrook, '35; Almquist, '41; Evans, Carver, and Draper, '44). The data presented here indicate that it may be of use in evaluating vegetable protein concentrates if the products are not overcooked. The three expeller process soybean oil meals and the two cottonseed meals appear to have been definitely overlooked as shown by the high content of residual protein. The same relation between protein quality index and supplementary protein nutritive value does not apply to the vegetable protein concentrates as it does to the animal concentrates. The lowest chemical protein quality index obtained in this experiment was 70.7 for cottonseed meal. The Alaska peas had a chemical protein quality index of about 80 and gave a gain in weight of 4.7 gm. per gram of supplementary protein consumed. Evans, Carver, and Draper ('44) obtained similar values for the chemical protein quality index of commercial herring fish meal, but the gain in weight per gram of supplementary protein consumed was 8.3 gm. One dogfish meal having a chemical protein quality index of only 44.5 gave a gain in weight of 5.7 gm. per gram of supplementary protein consumed. It is therefore evident that animal protein concentrates cannot be compared with vegetable protein concentrates by the chemical protein quality index proposed by Almquist, Stokstad, and Halbrook ('35).

The chemical protein quality index was not a satisfactory means of detecting differences in supplementary nutritive value between different soybean oil meals. It was first believed that the differences in supplementary nutritive value of different soybean oil meals were due to dif-

ferences in amino acid content of the meals. Since methionine has been shown to be the amino acid most likely to be lacking in soybean oil meals (Almquist, Mecchi, Kratzer, and Grau, '42), the sulfur distribution in these meals was determined. No differences were observed, however, in the cystine or methionine contents of the meals (Evans, '45 b) or in the per cent of organic sulfur dissolved by digestion with pepsin (Evans, '45 a).

It was found in the present study that there was a considerable difference between the different soybean oil meals with regard to the per cent of protein peptized by the different solvents. The cooked meals contained only one-tenth as much albumin as the uncooked meals. The heat-treated soybean oil meals having the highest percentage of glutelin gave the greatest gains in weight per unit of supplementary protein. Those having the least glutelin gave the least gains.

The differences in supplementary nutritive values of the different soybean oil meals appear to have been due to differences in degree of denaturation of the soybean proteins by heat treatment. The per cent of protein in the glutelin fraction was increased by short periods (up to 15 minutes) of autoclaving at 121°C., but was decreased by more prolonged heating. The curve obtained was very similar to the one presented by Bird and Burkhardt ('43) relating time of autoclaving to chick weight at 9 weeks of age. The effect of heat on the per cent of glutelin and the protein nutritive value is being further investigated. How heating and denaturation of the protein influences protein availability for the growing chick has yet to be determined, but is being investigated.

SUMMARY

A comparison was made between gain in weight per unit of supplementary protein as determined by Draper and Evans ('44) on ten samples of commercial soybean oil meal, two of cottonseed meal, and two of cull peas, and the chemical protein quality index (Almquist, Stokstad, and Halbrook, '35). A comparison between the gain in weight of chicks per unit of protein and the per cent protein present as albumin, globulin, prolamine, glutelin, and residual protein was also made.

The chemical protein quality index appeared to give good indication of the relative protein nutritive value of the vegetable protein concentrates studied with the exception of the overcooked soybean oil meals. A coefficient of correlation between the gain per unit of supplementary protein and the chemical protein quality index of + 0.946 was obtained

for all of the concentrates studied except the three expeller process soybean oil meals.

A high coefficient of correlation of + 0.928 between the gain per unit of supplementary protein and the per cent glutelin was obtained for the heat treated concentrates. A determination of the per cent glutelin may offer a chemical means of estimating differences in the protein nutritive value of soybean oil meals and, in combination with the per cent residual protein, the extent of heat denaturation of the soybean proteins.

LITERATURE CITED

ALMQUIST, H. J. 1941 Chemical estimation of quality in animal protein concentrates. J. Nutrition, vol. 21, p. 347.

ALMQUIST, H. J., E. MECCHI, F. H. KRATZER AND C. R. GRAU 1942 Soybean protein as a source of amino acids for the chick. J. Nutrition, vol. 24, p. 385.

ALMQUIST, H. J., E. L. R. STOKSTAD AND E. R. HALBROOK 1935 Supplementary values of animal protein concentrates in chick rations. J. Nutrition, vol. 10, p. 193.

BIRD, H. R., AND G. J. BURKHARDT 1943 Factors affecting the nutritive value of soybean oil meals and soybeans for chickens. Maryland Agr. Expt. Sta., Bull. A27, p. 35.

DRAPER, C. I., AND R. J. EVANS 1944 Plant protein concentrates in the chick ration. Poultry Sci., vol. 23, p. 507.

EVANS, R. J. 1945 a The sulfur content of the pepsin digestible fraction of protein concentrats. Arch. Biochem., vol. 7, p. 33.

———— 1945 b Unpublished results.

EVANS, R. J., J. S. CARVER AND C. I. DRAPER 1944 A comparison of the chemical protein quality index with the gross protein value of fish protein concentrates. Arch. Biochem., vol. 3, p. 337.

LUND, A. P., AND W. M. SANDSTROM 1943 The proteins of various tree seeds. J. Agr. Research, vol. 66, p. 349.

OLSON, F. C., AND L. S. PALMER 1940 Comparison of a chemical and a biochemical method for determining the biological value of proteins and an evaluation of the endogenous nitrogen. J. Agr. Research, vol. 60, p. 331.

OSBORNE, T. B. 1924 The Vegetable Proteins. Longmans, Green and Co., London.

OBSERVATIONS ON MONOMETHYLAMINOETHANOL AND DIMETHYLAMINOETHANOL IN THE DIET OF CHICKS

T. H. JUKES, J. J. OLESON AND A. C. DORNBUSH
Lederle Laboratories, Pearl River, New York

(Received for publication June 2, 1945)

It was noted recently (Jukes and Oleson, '45) that dimethylaminoethanol had a choline-like nutritional effect in promoting growth and preventing perosis in chicks on a diet deficient in choline. The present communication reports experiments with mono- and di- methylaminoethanol as related to growth and the prevention of perosis in chicks.

EXPERIMENTAL

Day-old New Hampshire Red chicks were placed in electrically-heated battery brooders and were fed the experimental diets immediately. Ten chicks were used in each group. The chicks were weighed weekly and were examined for perosis at frequent intervals. Basal diet 1 had the following composition: Glucose,[1] 56 parts; alcohol-extracted casein, 18; dried yeast,[2] 5; corn oil,[3] 5; gelatin, 5; gum arabic, 5; salt mixture ($CaCO_3$, 23 parts; K_2HPO_4, 10; KH_2PO_4, 10; NaCl, 17; bone ash, 30; $MgSO_4$, 5; ferric citrate, 3.5; $MnSO_4 \cdot 4H_20$, 1; copper carbonate, 0.1; zinc carbonate, 0.1; $Al_2(SO_4)_3 \cdot 18H_2O$, 0.5; KI, 0.8; cobalt acetate, 0.005; nickel carbonate, 0.002) 5. The birds were dosed three times weekly with 0.1 ml. of a solution of fat-soluble vitamins in corn oil. Each 0.1 ml. contained vitamin A, 7000 units; vitamin D_3,[4] 400 units; mixed tocopherols (34% solution), 20 mg.; 2-methyl-1, 4 naphthoquinone, 5 μg.

In the first experiment various supplements were added to the diet as summarized in table 1. The weights of choline refer to the chloride, of betaine to the hydrochloride and of mono- and di- methylaminoethanol to the free bases,[5] which were neutralized with hydrochloric acid before adding them to the diets.

[1] Cerelose.
[2] Anheuser-Busch, Strain G.
[3] Mazola.
[4] Delsterol.
[5] A sample of monomethylaminoethanol was kindly synthesized by Dr. J. Semb, and a second sample was furnished by the American Carbon and Carbide Company.

The observations in table 1 indicate that both monomethylaminoethanol and dimethylaminoethanol were effective in preventing perosis. Growth was stimulated by dimethylaminoethanol but not by monomethylaminoethanol. Methionine at the level fed was completely ineffective in preventing perosis or promoting growth as previously noted (Jukes, '41) but methionine stimulated growth when fed in combination with either monomethylaminoethanol or dimethylaminoethanol. There was an indication that methionine might stimulate growth slightly when added to the basal diet plus 0.2% choline. The possibility of a slight deficiency of methionine in diet 1 is not excluded upon comparing its probable methionine and cystine content with the

TABLE 1

Effect on growth and perosis in chicks produced by adding various compounds to basal choline-deficient diet 1.

ADDITION TO 100 GM. BASAL DIET 1	INCIDENCE OF PEROSIS AT		GAIN IN 28 DAYS
	21 days	28 days	
	%	%	gm.
None	60	80	113
0.2 gm. methionine	90	90	112
0.2 gm. monomethylaminoethanol	0	0	89
0.2 gm. methionine plus 0.2 gm. monomethylaminoethanol	0	0	123
0.2 gm. dimethylaminoethanol	0	0	147
0.2 gm. methionine plus 0.2 gm. dimethylaminoethanol	0	0	181
0.2 gm. choline	10	10	184
0.2 gm. methionine plus 0.2 gm. choline	0	0	196

requirement of the chick for these amino acids (Grau and Almquist, '43). The "choline" content of diet 1 was found to be approximately 0.018% by the Neurospora assay method (Horowitz and Beadle, '43).

A diet deficient in choline and methionine (diet 2) was prepared by modifying diet 1 as follows: 23 parts of "Alpha protein" [6] was substituted for the gelatin and casein, the yeast was reduced to 3 parts, the glucose was increased to 59 parts and the following synthetic vitamins were added per 100 gm. of diet: Thiamine, 1 mg.; riboflavin, 1 mg.; pyridoxine, 1 mg.; niacinamide, 5 mg.; calcium pantothenate, 10 mg.; inositol, 50 mg.; and biotin, 10 µg. "Alpha protein," derived from soybeans, has been described by Grau and Almquist ('43) as ". . . con-

[6] A commercial soya protein furnished by the Glidden Company.

taining 86.6% protein (N × 6.25) and 1.75% ash. The protein contained 1.53 ± 0.08% methionine and less than 0.25% cystine.'' This protein was used in place of casein in the diet in order to reduce the methionine content.[7] The effect of adding various supplements to this diet is summarized in table 2. Growth was not stimulated by dimethylaminoethanol unless methionine was also added. Perosis was not produced in chicks on the basal diet, but appeared in chicks which received the basal diet plus methionine. The results indicate that methionine was the limiting dietary factor when diet 2 was fed to chicks, but growth on diet 2 was not rapid even when this diet was supplemented with both methionine and choline.

TABLE 2

Effect on growth and perosis in chicks produced by adding various supplements to a diet deficient in choline and methionine (diet 2).

ADDITION TO 100 GM. BASAL DIET 2	INCIDENCE OF PEROSIS AT		GAIN IN 28 DAYS
	21 days	28 days	
	%	%	gm.
None	0	0	27
0.2 gm. dimethylaminoethanol	0	0	20
0.2 gm. choline	0	0	37
0.6 gm. methionine	60	60	108
0.6 gm. methionine plus 0.2 gm. dimethylaminoethanol	0	0	127
0.6 gm. methionine plus 0.2 gm. choline	0	0	139

DISCUSSION

It is apparent that differences exist between the rat and the chick with regard to the utilization of certain precursors of choline. It has been observed with rats that ethanolamine (Stetten, '41) may function as a ''methyl acceptor'' for the biological synthesis of choline, and that ethanolamine may be formed from glycine (Stetten, '41) or serine (Stetten, '42). Methionine (du Vigneaud, '42–'43) or betaine (Simmonds et al., '44) may donate methyl groups to aminoethanol in the biological synthesis of choline in the rat. In the case of chicks fed certain purified diets, it has been found that choline deficiency is not alleviated by supplements of betaine, methionine or methionine plus aminoethanol (Jukes, '41; Jukes and Welch, '42; Almquist and Grau, '44). However it was found by McGinnis and co-workers ('44) that betaine or methionine would relieve choline deficiency in chicks fed certain diets of natural foods.

[7] Diet 2 was found to give a response corresponding to about 0.010% of choline and ''Alpha protein'' to about 0.009% of choline when assayed by the Neurospora method.

It has been shown (Horowitz and Beadle, '44) that mutant no. 34486 of Neurospora crassa is unable to grow in a purified culture medium, but will grow if a few micrograms of choline are added. These workers also observed that mutant no. 34486 is unable to use betaine or aminoethanol as substitutes for choline and that methionine has very little effect. Recently it has been found (Jukes, Dornbush and Oleson, '45) that the growth-stimulating power of betaine, aminoethanol and methionine for this mutant was not increased by using combinations of aminoethanol and betaine, or aminoethanol and methionine. It may be postulated that mutant no. 34486 has lost the ability, which the wild strain of Neurospora crassa may be presumed to possess, to methylate aminoethanol.

It has also been observed that the chick (Jukes and Oleson, '45), like mutant no. 34486, can utilize dimethylaminoethanol in place of choline (Jukes and Dornbush, '45). These various observations lead to the postulation that dimethylaminoethanol represents an intermediate step in the formation of choline by the "methylation" of aminoethanol.

The results in table 1 indicate that monomethylaminoethanol is utilized by the chick similarly to dimethylaminoethanol but less efficiently, as though monomethylaminoethanol were possibly a precursor of dimethylaminoethanol. A preceding step may be the formation of monomethylaminoethanol from aminoethanol and a "methyl donator". This step is apparently not accomplished by the chick as judged by the ineffectiveness of a supplement of methionine plus aminoethanol (Jukes, '41).

A response differing from those observed with the rat and the chick has been reported for a strain of Type III pneumococcus (Badger, '44). This organism can utilize ethanolamine, which it appears to be unable to synthesize, in place of choline in a basal medium containing methionine. A large number of substituted ethanolamines were also active.

SUMMARY

1. Monomethylaminoethanol and dimethylaminoethanol were found to prevent perosis in chicks when added to a purified diet which was deficient in choline. Growth was promoted by dimethylaminoethanol but not by monomethylaminoethanol.

2. The addition of methionine to the diet did not stimulate growth but growth was increased when methionine was added to the basal diet plus monomethylaminoethanol or to the basal diet plus dimethylaminoethanol.

3. Experiments were also made with a basal diet deficient in both methionine and choline. Growth was not stimulated by dimethylaminoethanol unless methionine was also added. Chicks receiving the basal diet did not develop perosis, but perosis developed in chicks receiving the basal diet plus methionine.

4. These observations are compared with certain results which have been reported in experiments with the rat and with the "cholineless" mutant no. 34486 of Neurospora crassa.

LITERATURE CITED

ALMQUIST, H. J., AND C. R. GRAU 1944 Interrelation of methionine, choline, betaine and arsenocholine in the chick. J. Nutrition, vol. 27, p. 263.

BADGER, E. 1944 The structural specificity of choline for the growth of type III pneumococcus. J. Biol. Chem., vol. 153, p. 183.

DU VIGNEAUD, V. 1942–1943 The significance of labile methyl groups in the diet and their relation to transmethylation. The Harvey Lectures Series 38, pp. 39–62.

GRAU, C. R., AND H. J. ALMQUIST 1943 The utilization of the sulfur amino acids by the chick. J. Nutrition, vol. 26, p. 631.

HOROWITZ, N. H., AND G. W. BEADLE 1943 A microbiological method for the determination of choline by use of a mutant of Neurospora. J. Biol. Chem., vol. 150, p. 325.

JUKES, T. H. 1941 The effect of certain organic compounds and other dietary supplements on perosis. J. Nutrition, vol. 22, p. 315.

JUKES, T. H., AND A. C. DORNBUSH 1945 Growth stimulation of Neuropsora cholineless mutant by dimethylaminoethanol. Proc. Soc. Exp. Biol. and Med., vol. 58, p. 142.

JUKES, T. H., A. C. DORNBUSH AND J. J. OLESON 1945 Unpublished observations.

JUKES, T. H., AND J. J. OLESON 1945 Choline-like nutritional effect of dimethylaminoethanol. J. Biol. Chem., vol. 157, p. 149.

JUKES, T. H., AND A. D. WELCH 1942 The effect of certain analogues of choline on perosis. J. Biol. Chem., vol. 146, p. 19.

MCGINNIS, J., L. C. NORRIS AND G. F. HEUSER 1944 Influence of diet on chick growth-promoting and antiperotic properties of betaine, methionine and choline. Proc. Soc. Exp. Biol. and Med., vol. 56, p. 197.

SIMMONDS, S., MILDRED COHN, J. F. CHANDLER AND V. DU VIGNEAUD 1944 Further studies of transmethylation with deuterium. Abstracts of Papers, 108th Meeting, American Chemical Society, p. 49B.

STETTEN, D., JR. 1941 Biological relationships of choline, ethanolamine, and related compounds. J. Biol. Chem., vol. 140, p. 143.

——— 1942 The fate of dietary serine in the body of the rat. J. Biol. Chem., vol. 144, p. 501.